MAY 25 1920

THE JOURNAL

OF

GENERAL PHYSIOLOGY

EDITORS

JACQUES LOEB W. J. V. OSTERHOUT

VOLUME II, NO. 5 MAY 20, 1920

PUBLISHED BIMONTHLY

AT MOUNT ROYAL AND GUILFORD AVENUES, BALTIMORE, MD.

BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

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CORRECTION.

On page 243, Vol. ii, No. 3, January 20, 1920, line 12 (counting each equation as a line) for *a photochemical reaction* read ***a reversible photochemical reaction.***

THE EFFECT OF TEMPERATURE UPON FACET NUMBER IN THE BAR-EYED MUTANT OF DROSOPHILA.*

PART II.

BY JOSEPH KRAFKA, JR.

(*From the Zoological Laboratory of the University of Illinois, Urbana.*)

(Received for publication, December 30, 1919.)

Determination of the Period during Which Temperature is Effective in Modifying the Facet Number.

Preliminary Experiments on Unselected Bar Stock at 30 and 15°.

A preliminary experiment was planned to determine whether temperature had an effect throughout the immature stages or whether it was limited to a specific phase of development. In Table XIV[1] are shown the results of subjecting successive cultures for the 1st day, first 2 days, first 3 days, and so on to 30° before subsequent development at 15°. 3, 2, and 1 days at 30° give the same facet number as those raised at 15° throughout. The early days of larval life may be spent at high temperatures without effect on the facet number.

6 and 7 days at 30° followed by transfer to 15° show that the number of facets had been determined prior to the transfer, as indicated by the fact that all the counts come well within the range of the stock counts at 30°.

Next, if we consider only those counts made on the 1st day of hatching, the bottles that were kept at 30° for the first 4 and 5 days show only the facet counts characteristic of the 30° stocks. The counts made on the flies hatching on the 2nd and 3rd days are inter-

* Contribution from the Zoological Laboratory of the University of Illinois, No. 148.

[1] Krafka, J., Jr., *J. Gen. Physiol.*, 1919-20, ii, 409.

mediate between the 30 and 15° stocks. These intermediates were to be expected. The parents were not removed from the bottles until the end of the first 24 hours. Hence some of the larvæ may have been 24 hours older than others. Those 4 days old had already passed the point X in development during which the facet number-determining reaction is going on. They are also the first individuals in the bottle to hatch. Those hatching later were not so far along in development and hence were affected by the transfer to 15°.

Obviously temperature is not capable of modifying the facet number throughout the immature stages, but is limited to a definite stage in development.

Subsequent experiments on Ultra-bar consider these points more in detail.

Effect of Temperature during the Pupal Period.

Experiments of the same type as the preceding were carried out on Ultra-bar at 27 and 15°. We will consider the effects of temperature during the pupal period first. Subjecting the cultures to 27° for 5, 6, 7, 8, and 9 days before subsequent removal to 15° gave counts which are characteristic of the 27° stocks both in mean and in individual variation. The first four cultures had pupated before the transfer to the colder temperature was made. In the last one the imagos had begun to emerge from pupation as the transfer from 27 to 15° was made. Obviously in the last case a change of temperature would have no effect, since there is no further change in the number of facets after the adult is once formed.

From the distribution of the counts in Table XV it is just as clear that subjecting the immature stage to low temperature after pupation has no effect on the facet number. This is a particularly important point, since the facets themselves first become evident only toward the end of the pupal period. The reaction which has determined the number of facets that are to form has greatly preceded the actual formation of these facets (Fig. 11).

TABLE XV.

Experiment 45, Showing the Distribution of Facet Numbers. Larvæ Were Allowed to Complete Part Development at 27° and Were then Transferred to 15° for the Remainder.

No. of days at 27° before transfer to 15°.....	1		2		3		4		5		6		7		8		9	
Facet class.	Distribution of individuals in separate bottles.																	
	Larval period.								Pupal period.									
7–10	*	*					4	1		1								
11–14							4	2	7	2	2							
15–18						1	8	6	15	8	4	1	7		4	1	6	3
19–22							23	11	19	17	9	5	21	9	14	9	14	6
23–26					2		8	12	7	7	9	15	17	18	21	28	16	17
27–30					7	3	2	6		3		2	2	8	5	12	2	7
31–34					3	3	6	7								1		1
									Upper range of 27° stock counts.									
35–38					6	3	2	2										
39–42		1	2		8	4												
									Lower range of 15° stock counts.									
43–46	5	0	16	2	8	7												
47–50	3	1	12	7	3	4												
51–54	1	2	11	10	3	8												
55–58	1	2	8	16	1	7												
59–62	1	4	1	5		2												
63–66	1	0		6		1												
67–70		1		2		1												
71–74				2		1												
Total No. of individuals....	12	11	50	50	41	45	57	47	48	38	24	23	47	35	44	51	38	34

* The first column represents females; the second, males.

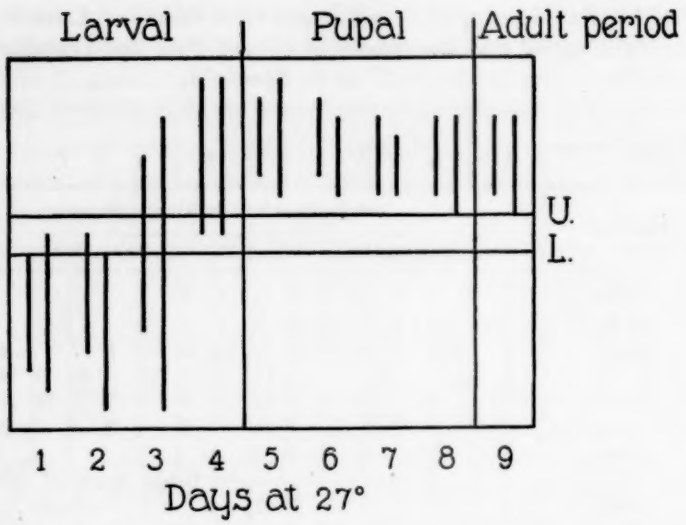

FIG. 11. Effect on the distribution of facet counts as a result of subjecting successive cultures from the same parentage of Ultra-bar to varying periods of time at 27° before subsequent development at 15°. After mating the first bottle was put at 27° for 24 hours. The parents were then removed to the second bottle. The first bottle was allowed to complete development at 15°. At the end of 24 hours, the parents were removed to a third bottle, etc. The second bottle remained at 27° for 48 hours, and completed development at 15°. The third bottle remained at 27° for the first 3 days, etc. The first vertical line of a pair represents the range of the female counts, the second is that of the males. Those cultures to the left of the first full vertical line were in the larval stage when the transfer from 27 to 15° was made. Those between the two full vertical lines were in the pupal stage when the transfer was made (see Table XV). U. is the upper range of 27° stock counts; L., the lower range of 15° stock counts.

Effect of Temperature during the Larval Period. Initiation and Duration of the Effective Period at 27°.

An examination of Table XV shows that a temperature of 27°C. for 1 or 2 days at the beginning of the larval period has no effect on the facet number, as only three individuals out of 123 are slightly outside the range of the stock experiments at 15°.

Of those that passed the first 3 days at 27°, some had developed beyond the point X, at which facet number is determined, since their counts are characteristic of the 27° stock. Some were just in the effective period when the transfer from 27 to 15° was made, as shown by the fact that the counts are intermediate between those characteristic of the 27 and those of the 15° stocks. Some individuals were not quite so far along and hence the whole facet number-determining reaction was carried out at 15°.

4 days at 27° brought nearly all individuals through the effective period. Four individuals out of 104 are slightly above the upper range of the 27° stock counts. We may conclude that the stage in development, at which the facet number is being determined, is passed prior to the end of 4 days at 27°. Likewise this stage is not reached by the end of 2 days at 27°.

To define this period more closely Experiment 59 was planned. After many unsuccessful attempts to get a series in which the eggs were not more than 1 hour apart in age, the present series was carried through. Here the parents were allowed to lay eggs during a period of 12 hours. This series includes the following number of days at 27° with subsequent removal to 15°; 1 day (24 hours), 2, $2\frac{3}{4}$, 3, $3\frac{1}{4}$, $3\frac{1}{2}$, $3\frac{3}{4}$, and 4 days. The results are given in Table XVI and in Fig. 12.

This experiment bears out the previous one in that 1, 2, $2\frac{3}{4}$, and 3 days at 27° did not bring the larvæ up to the point X. This phase of development is initiated between 3 and $3\frac{1}{4}$ days as shown by the intermediate condition of the counts of the latter. These are predominated by the lower temperature. $3\frac{1}{2}$ days at 27° give a preponderance of individuals with the 27° count. $3\frac{3}{4}$ days at 27° have brought all but one individual through the effective period. After 4 days at 27°, all individuals had completed this stage in de-

velopment and further changes in temperature had no effect on the facet number thereafter.

A second culture, allowed to develop 3 days at 27°, demonstrates individual variation in rate among separate bottles. In this culture the effective period was passed by thirteen individuals at 27°,

TABLE XVI.

Experiment 59, Showing Distribution of Facet Numbers. Larvæ Were Allowed to Complete Part Development at 27° and Were then Transferred to 15° for Remainder.

No. of days at 27° before transfer to 15°.	1		2		2½		3 (I)		3 (II)		3½		3¾		4		27° Control.	
Facet class.	Distribution of individuals in separate bottles.																	
15–18											5		2		3		6	3
19–22									2		3	1	8	4	8	5	36	23
23–26									3		7	3	11	11	11	14	37	38
27–30									4		4	10	3	7	3	11	19	32
31–34	Upper range of 27° stock.								2	2	8	5		1			4	3
35–38									4	7	12	4		1				
39–42					1		1		9	5	12	9	Lower range of 15° stock.					
43–46	1		1	2	1	1			7	3	3	5						
47–50	4		6	2	4	1	9		9	4	2	6						
51–54	17	2	11	3	9	11	9	3	13	7	2	5						
55–58	12	7	4	4	8	9	5	1	1	3	3	1						
59–62	6	7	6	6	6	5	2	4	2	4	1	0						
63–66	4	7	2	3	3	6	2	11	2	3		1						
67–70	3	10	2	5		2	1	7		3	1							
71–74	3	5		1				4		2								
75–78		2																
79–82								1										

before the transfer was made; and in many others the counts are intermediate showing that the reaction was going on at the time of the transfer from 27 to 15°.

A control mating by the same parentage as the foregoing series was reared at 27°. It shows the normal distribution of the 27° stock.

According to this and the previous experiment, the reaction, which determines the number of facets and whose rate is subject to temperature modification, is initiated at 27° at or near the end of 3 days of development. Furthermore, it is practically over at the end of 3¾ days, making the effective period of less than 18 hours duration.

The actual time during which the reaction which determines the number of facets takes place is doubtless much shorter than this for

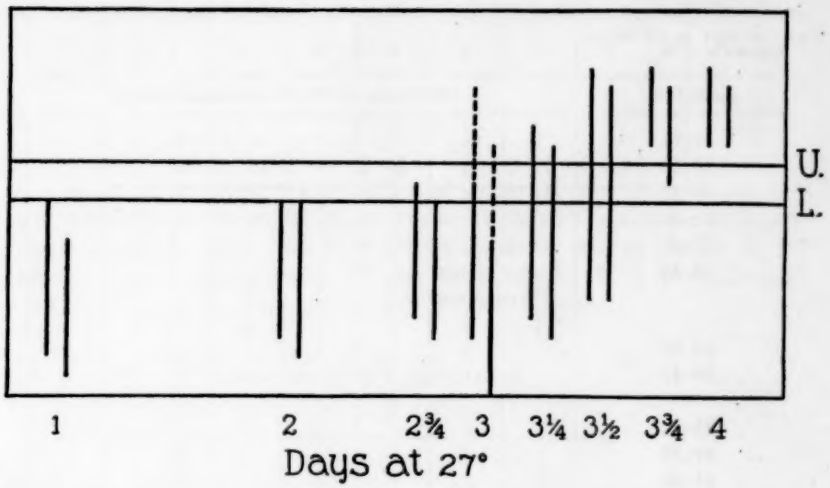

FIG. 12. Effect on the distribution and range in the Ultra-bar as a result of subjecting successive bottles to 27° for 1, 2, 2¾, 3, 3¼, 3½, 3¾, and 4 days, respectively, before removal to 15° (see Table XVI). Broken line represents extension of range in a second culture kept at 27° for 3 days. U. is the upper range of 27° stock counts; L., the lower range of 15° stock counts.

the individual, since under the conditions of the experiment we are dealing with material that may differ 12 hours in age.

Initiation and Duration of the Effective Period at 15°.

Experiment 62, of the same type as the preceding except that the conditions were reversed, was carried out to determine when the effective period was initiated at 15°. The same parents were used as in Experiment 59. The cultures were made up at 27° and left

for 24 hours. The parents were then removed to the next set of bottles, while the bottles containing the eggs and larvæ were transferred to 15°. Here they were left for the number of days indicated in Table XVII and then returned to 27° to complete development.

TABLE XVII.

Experiment 62, Showing Distribution of Facet Numbers. Larvæ Were Allowed to Complete Part of Development at 15° and Were then Transferred to 27° for Remainder.

No. of days at 15° before transfer to 27° *	1		2		3		4		5		6		7		8	
Facet class.	Distribution of individuals in separate bottles.															
11–14			4						2		1		1			
15–18	5	1	8		5	1	7	2	15	5	13	7	3	1		
19–22	15	16	35	16	25	25	23	15	18	21	17	22	4	6		
23–26	23	26	50	63	15	18	26	32	6	18	8	7	3	1		
27–30	2	12	7	21	3	9	4	12		2	1	1		1		
31–34	Upper range of 27° stock counts.					2		1							1	
35–38																
39–42	Lower range of 15° stock counts.														1	
43–46															1	
47–50															1	
51–54															1	
55–58																
59–62																1
63–66																
67–70																1
71–74																
75–78																
79–82																

* The bottles were at 27° the first 24 hrs.; then removed to 15° for the number of days indicated before return to 27°. Parents are the same as in Experiment 59.

It is plainly evident that the effective period is not initiated at the end of 3 days as at 27°. The point X is not reached even at the end of 7 days at 15° plus the 1st day at 27°. In Experiment 62, the change comes on the 8th day at 15° (Fig. 13). Unfortunately this experiment was not planned to cover a longer period; a second experiment was therefore started.

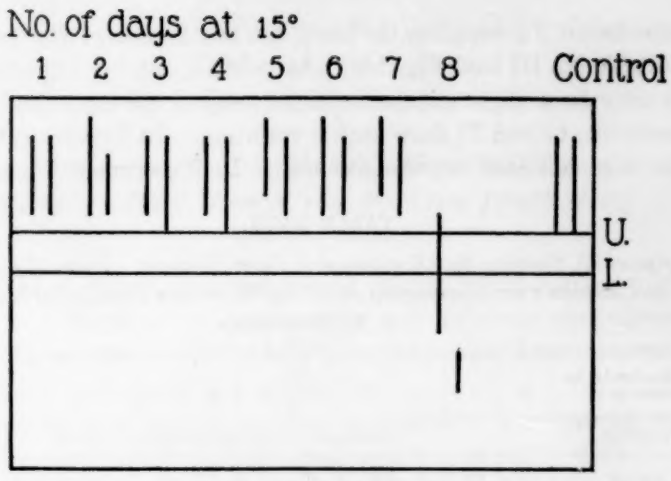

FIG. 13. Effect on the range of facet counts in Ultra-bar as a result of subjecting successive cultures from the same parents for varying periods to 15° before subsequent development at 27° (see Table XVII). U. is the upper range of 27° stock counts; L., the lower range of 15° stock counts.

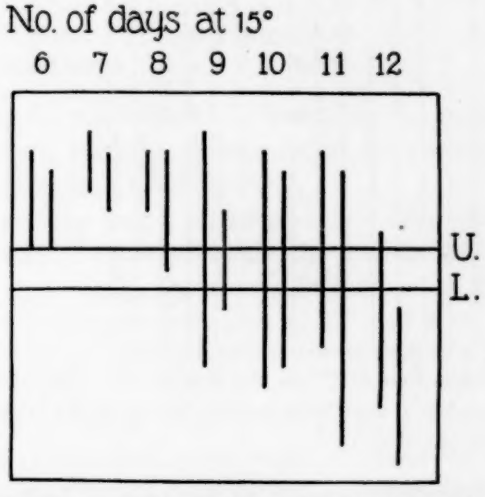

FIG. 14. Duration of the effective period at 15°. Effect on the range of facet counts as a result of subjecting successive cultures from the same parentage for varying periods of time to 15° before subsequent development at 27° (see Table XVIII). U. is the upper range of 27° stock counts; L., the lower range of 15° stock counts.

Experiment 72 supplies the data on the length of the period at 15° (Table XVIII and Fig. 14). As pointed out for Experiment 59 there may be a slight difference in the rates of the individual bottles. Experiments 62 and 72 show such a variation. In Experiment 62, the period was initiated on the 8th day. In Experiment 72, only two

TABLE XVIII.

Experiment 72, Showing the Distribution of Facet Numbers. Larvæ Were Allowed to Complete Part Development at 15° and Were then Transferred to 27° for the Remainder.

No. of days at 15° before return to 27°.*..	6		7		8		9		10		11		12		
Facet class.	Distribution of individuals in separate bottles.														
11–14			1				2								
15–18	1		2	3	2	1	1		1		1				
19–22	6	6	4	4	5	3	0		3	1	1	1			
23–26	1	2		2	5	1	2		3	4	3	2			
27–30						2	4	4	2	3	4	3			
31–34	1	1				2	3	2	1	3	2		1		Upper range of 27° stock.
35–38							1	3	1	1	3		1		
39–42						1	2		2	1	2	1	3		Lower range of 15° stock.
43–46							1	1	1		2	3	7		
47–50										2	1		8	3	
51–54									2	1	2	2	16	7	
55–58							1			2		1	6	8	
59–62									1			2	3	14	
63–66													1	6	
67–70						1						1		8	
71–74												1		1	
75–78														1	

* All these cultures were at 27° for the first 24 hrs. They were then removed to 15° for the number of days indicated at the top of the table and allowed to complete development at 27°.

individuals had passed the effective period at 15° when the transfer was made from 15 to 27°. After 9, 10, and 11 days an increasing number of individuals show the 15° count, and after 12 days only five of 94 individuals had not completed the facet number-determining reaction when the transfer was made.

As there may be 24 hours difference in age among the individuals in a bottle, the period at 15° is practically limited to the 9th, 10th, and 11th days, or the length of the period at 15° is about 72 hours.

Comparison of the Length of the Period during Which Temperature is Effective on Facet Number with the Total Length of the Immature Stage.

It has been shown that the period during which temperature is effective is initiated at two very remote time intervals when development is carried out at 15 and 27°, respectively. Does this period represent a definite stage in development?

The total number of days required to complete development of the immature stages at 15° is 31.87, while at 27° it is 9.21. This gives a daily rate of 3.13 per cent total development at 15° and 10.86 per cent total development at 27°. With these rates we may calculate the point X.

3 days at 27°	3 × 10.86 =	32.58	per cent
8 " " 15°	8 × 3.13 =	25.04	" "
1 day " 27°	1 × 10.86 =	10.86	" "
		35.90	" "

In other words, the reaction which determines the number of facets that will develop in the adult is initiated at the completion of 32 to 36 per cent of immature development.

Comparing now the length of the periods during which this reaction is going on at 15 and at 27° with the total length of the immature stages at those temperatures, we find 18 hours at 27° and 72 hours at 15° as opposed to 9.21 days at 27° and 31.87 days at 15°. These ratios are a fair approximation considering the experimental conditions.

Expressing the length of the effective period in percentage of the total development and adding the results to those for the initiation of the period, we find that the reaction which determines the number of facets starts at the completion of 32 per cent of development and ends with the completion of 45 per cent.

Thus the length of a particular phase of development is proportional to that of any other phase. Reciprocally, the rate of a given

reaction such as that which determines facet number is proportional to the rate of general metabolism.

In the first part of this study we noted a correlation between facet number and length of the immature stage when the latter was completed at a single temperature. Subsequently it appeared that facet number and the total length of the immature period were independent. Why do we find this apparent correlation?

The facet-determining reaction has been shown to be of relatively short duration. It is obvious that a change in temperature following its completion could affect the total length of the immature period without affecting the facet number.

We may conclude that the number of facets and the length of the immature period are not directly dependent but rather that the former is determined by a specific reaction the rate of which is correlated with that of the general metabolic processes extant while this particular phase of development is going on.

THE EFFECT OF TEMPERATURE UPON FACET NUMBER IN THE BAR-EYED MUTANT OF DROSOPHILA.*

PART III.

BY JOSEPH KRAFKA, JR.

(From the Zoological Laboratory of the University of Illinois, Urbana.)

(Received for publication, December 30, 1919.)

The Effect of Temperature upon Variability.

Coefficient of Variability at Different Temperatures.

This study gives some interesting data on the question of individual variability at the various temperatures. The distribution of the counts of Ultra-bar are given in Table XIX. Here the class size is 1 facet. Particular attention is called to the normal distribution in all cases.

An argument for the genetic stability of the Ultra-bar stock is found in the relatively infrequent occurrence of individuals outside the bounds of normal distribution. At 27° a single 55 facet male and at 16° a 72 facet male are the only two extremely wide departures.

The mean, standard deviation, and coefficient of variability for all three stocks are given in Tables XX to XXII. Two things are apparent: (1) variability increases with the temperature when measured by the coefficient of variability; and (2) the variability of the Ultra-bar stock is much lower than that of the other two stocks. Both these generalizations have their exceptions. Their reliability as compared to statements made by other writers will be discussed later.

* Contribution from the Zoological Laboratory of the University of Illinois, No. 148.

TABLE XIX.

Showing the Distribution of the Facet Counts in the Stock Experiments on Ultra-Bar.

Facet No.	Temperature.																	
	15	16	17.5	20	23	25	27	29	31	15	16	17.5	20	23	25	27	29	31
	Females.									Males.								
5								1	1									1
6									1									1
7									1								1	4
8									2									4
9								2	3									6
10								5	4								6	8
11								8	6								1	15
12							2	14	14								9	20
13							5	19	16							1	10	16
14						1	2	46	21							1	17	26
15						2	11	74	19							1	36	22
16						2	11	63	15							1	40	22
17						1	22	77	11							8	55	16
18						5	38	76	10							20	84	7
19						5	58	72	8						1	26	96	9
20						14	59	52	2						6	34	83	3
21				1	1	26	66	48	2						7	53	82	3
22				2	1	44	61	29	0						14	67	55	2
23				1	5	32	38	17	1					2	23	59	25	1
24				3	8	61	38	12	1					1	27	55	20	1
25				2	9	59	28	10						3	54	55	9	
26				4	8	72	17	3					1	3	51	57	10	
27				2	7	58	15	2					0	3	58	30	2	
28				7	11	50	9						2	5	59	22		
29				6	10	38	8						4	11	59	21		
30				7	10	13							5	8	39	9		
31		1	3	12	7	8	1						6	5	45	8		
32		0	5	13	6	7							7	12	25	6		
33		2	10	8	3	2	1						7	15	12	2		
34		0	8	10	2								9	12	9	1		
35		3	9	6	3								9	5	5	0		
36		4	8	4	2							1	4	3	1			
37		3	12	6	1						2	2	12	3	1			
38		6	10	3			1				1	1	7	2				
39	1	10	11	5							0	2	8	1				
40	0	3	3	1							1	5	4					
41	1	8	7	4							3	9	14					
42	2	11	0	1						1	4	10	8					
43	4	7	5	0						0	3	10	6					
44	3	10	6	3						1	2	7	4					
45	3	5	3	0						1	1	10	0					

TABLE XIX—*Concluded.*

Facet No.	Temperature.																	
	15	16	17.5	20	23	25	27	29	31	15	16	17.5	20	23	25	27	29	31
	Females.									Males.								
46	3	3	0	0						0	0	8	1					
47	3	4	2	1						0	6	9	0					
48	3	4	4							0	10	11						
49	5	2	3							2	7	2						
50	1	2	1							1	5	1						
51	1	3	0							2	9	1						
52	2	2	1							3	9	1	1					
53	2	2	1							3	6	4						
54	5	2								2	4	3	1					
55	2	0								2	7	1				1		
56	1	1								0	4	0						
57	1	1								5	5	1						
58	3	1								4	5	0						
59	0	0								4	2	1						
60	1	0								2	1							
61	1	1								3	3							
62	2									4	1							
63	0									4	1							
64	0									2								
65	1									0								
66	1									1								
67	1									4								
68	0									3								
69	1									1								
70	0									0								
71	0									3								
72	1									1	1							
73	0									0								
74										1								
75										1								
76										1								
77										1								
78																		
79																		
80																		
81																		
82																		
83										1								
84																		
85										1								

TABLE XX.

Coefficient of Variability at the Various Temperatures. Unselected Bar Stock. Females.

Temperature.	Mean facet No.	Standard deviation.	Coefficient of variability.
°C.			
15	213.67 ± 2.12	29.78 ± 1.48	13.94
20	122.20 ± 1.46	23.45 ± 1.02	19.19
25	81.08 ± 1.21	19.19 ± 0.85	23.67
30	39.66 ± 0.87	12.73 ± 0.61	32.10

TABLE XXI.

Coefficient of Variability at Various Temperatures. Low Selected Bar Stock. Females.

Temperature.	Mean facet No.	Standard deviation.	Coefficient of variability.
°C.			
15	189.00 ± 2.26	29.77 ± 1.58	15.75
16	158.20 ± 1.56	23.58 ± 1.10	14.90
17.5	127.27 ± 1.49	22.19 ± 1.04	17.43
20	98.88 ± 1.27	19.23 ± 0.90	19.45
25	74.25 ± 0.87	16.55 ± 0.61	22.29
27	55.13 ± 0.73	13.88 ± 0.52	25.18
29	47.40 ± 0.63	9.31 ± 0.44	19.64
30	36.56 ± 0.79	9.37 ± 0.56	25.63
31	28.85 ± 0.41	6.07 ± 0.29	21.04

TABLE XXII.

Coefficient of Variability at Various Temperatures. Ultra-Bar Stock. Females.

Temperature.	Mean facet No.	Standard deviation.	Coefficient of variability.
°C.			
15	51.51 ± 0.70	7.69 ± 0.49	14.93
16	43.27 ± 0.39	5.83 ± 0.28	13.47
17.5	38.57 ± 0.32	5.03 ± 0.23	13.04
20	32.59 ± 0.32	5.06 ± 0.22	15.53
23	28.30 ± 0.24	3.52 ± 0.17	12.44
25	25.24 ± 0.09	3.12 ± 0.07	12.36
27	21.27 ± 0.10	3.35 ± 0.07	15.75
29	17.23 ± 0.08	3.23 ± 0.06	18.74
31	14.57 ± 0.18	3.16 ± 0.13	21.69

The Sex Coefficient at Different Temperatures.

A marked sexual difference exists in all these experiments. The average value for the ratio between the mean facet number of the females and that of the males is 0.791. Temperature has no consistent effect in altering this ratio.

An explanation of the sexual difference is to be sought in the fact that we are dealing with a sex-linked factor. On the chromosome hypothesis, a double dose of the restricting factor is present in the female while only a single dose is present in the male.

Inheritance of Temperature Effects.

Comparison of Offspring Raised at 27°, from Parents Reared at 15, 20, and 27°, Respectively.

The interest in this phase of the work is both practical and theoretical. To preclude any inherited effect in the stock experiments, care was taken to keep the parent stocks at 27°. To determine whether or not there was an inherited effect, Experiment 51 was designed. Flies reared for one generation in the stock experiments at 15, 20, and 27° were used as parents in cultures that were made up under conditions as nearly alike as possible. The offspring from the 15° parents, from the 20° parents, and from the 27° parents were thus allowed to develop simultaneously at 27°.

The distribution of the facet counts of the parents and the offspring is given in Table XXIII. The mean facet values and the standard deviations are given in Table XXIV. The mean facet number, the standard deviation, and the distribution of the F_1 stock reared at 27° are characteristic of the 27° stock counts although those of the parents are markedly different. There is no inherited effect of temperature upon facet number in this case.

TABLE XXIII.

Showing the Inherited Effect of Temperature. Distribution of Facet Number of Parents Reared at 15, 20, and 27°, Respectively, as Compared to Their Offspring All of Which Were Reared Simultaneously at 27°.

Facet No. classes.	Parents reared at.						Offspring reared at 27° from parents at.					
	15°		20°		27°		15°		20°		27°	
	Distribution of individuals.											
12–15					1				1		1	
16–18					7	2	1		4	2	3	2
19–21					13	6	4	3	26	11	15	7
22–24					10	11	11	15	12	28	10	14
25–27					4	9	10	14	5	15	1	8
28–30			4				2	5		1		
31–33			9					1				
34–36			6	6								
37–39	1		8	8								
40–42	1	1	2	12								
43–45	2		2	9								
46–48	2	3	1									
49–51	2											
52–54	7			2								
55–57	1	4										
58–60	1	1										
61–63	1	2										
64–66		3										
67–69		1										
70–72		1										
73–75		1										

TABLE XXIV.

Mean Facet Number and Standard Deviation of Parents Reared at 15, 20, and 27°, and of Their Offspring Reared at 27°.

Parents reared at.	Mean facet No.	Standard deviation.	Offspring reared at 27° from parents reared at.	Mean facet No.	Standard deviation.
°C.			°C.		
15	50.50 ± 0.96	6.05 ± 0.67	15	23.67 ± 0.35	2.76 ± 0.25
	58.94 ± 1.35	8.28 ± 0.95		24.85 ± 0.28	2.53 ± 0.20
20	35.50 ± 0.54	4.53 ± 0.38	20	20.85 ± 0.24	2.61 ± 0.18
	40.60 ± 0.48	4.29 ± 0.34		23.12 ± 0.24	2.80 ± 0.17
27	20.80 ± 0.34	3.02 ± 0.24	27	20.50 ± 0.29	2.39 ± 0.20
	22.96 ± 0.12	2.43 ± 0.08		22.68 ± 0.31	2.56 ± 0.22

DISCUSSION.

Temperature as a Factor in the Mechanism of Development.

Direct Effect of Temperature upon Growth, Size, Number of Parts, Structure, and Color.

Ordinarily temperature is not a factor capable of modifying structure to any marked extent. Certainly structural variations are in no way comparable with the variations in rate at which they are brought about. The capacity to develop specific color, size, and form is an heritable characteristic; *e.g.*, the present study involves the white-eyed mutant of *Drosophila*, of which all individuals are white-eyed regardless of the temperature at which they develop.

Many organisms, however, exhibit variations in their structural characteristics which may be considered as a direct response to temperature.

Among the earlier investigators of temperature effects on structure were Merrifield, Weismann, Standfuss, Fischer, and Dorfmeister.[1] The chief object of their experiments was the production by environmental manipulation of the various racial and polymorphic forms in the Lepidoptera.

Vernon (1895) found that the size relations between various parts of the echinoderm larvæ could be modified in response to different temperatures at which they developed. Standfuss (1895) found a reduction in the size of the imagos, as a result of rearing lepidopterous larvæ at high temperatures. This he ascribed to the indirect effect of insufficient nourishment.

Tower found that by subjecting larvæ of *Leptinotarsa decemlineata* to various temperatures he could affect the amount of pigmentation in the adult. His results are unique in that an increase from the mean temperature range of the species (22.5°C.) had the same result as a decrease. He obtained an increase in melanism down to 16° and up to 28° followed by a decrease to albinism beyond these temperatures.

Shelford found a tendency toward melanism with an increase in temperature due to the reduction in size of the unpigmented areas on the elytra of the tiger beetles.

All these reactions are complex and the materials do not lend themselves to close quantitative study. It is obvious that no simple temperature relations can be worked out for them.

[1] For a complete review, see Bachmetjew.

Examples in Which a Specific Structure Depends upon a Definite Environmental Stimulus.

In three well marked cases, specific temperatures determine the character of the organism.

Baur cites a case of *Primula sinensis* which at ordinary temperatures produces red flowers. If a plant is subjected to a temperature of 30–35°C. a few weeks before blooming, the flowers will be white. If the plant is returned to 15–20°C., the buds opening immediately will still be white but those developing later will be red. As Baur points out, white cannot be said to be inherited, red cannot be said to be inherited, but rather the capacity to produce red flowers at 15° and white ones at 30° is the thing inherited.

Hoge found a race of *Drosophila* in which one or more legs showed reduplication. Under ordinary cultural conditions only about 10 per cent of the individuals from a pure reduplicated mating showed the condition. It was later discovered that by subjecting the eggs to 9–10°C. the percentage of offspring showing the character could be raised to practically 100.

Roberts found that a temperature difference of 4–5° C. had a direct effect on the length of the wing in the mutant "vestigial" of *Drosophila*, much greater than twenty-nine generations of selection had produced.

Bar eye is a sex-linked factor that reduces the number of facets in the adult *Drosophila* from 1,000 to 70. Its Mendelian behavior is clear cut and regular. As shown in this paper, however, the number of facets produced, when this factor is present, is dependent upon the specific temperature at which a definite stage in larval development is passed.

In the first three examples only two temperatures are involved. Obviously no quantitative measure can be applied although the results are very definite.

In bar eye, observations have been made at close intervals over a range of temperature from 15–31°C. The temperature relations have been shown to approximate closely those of many physiological reactions.

In certain cases other environmental factors may be said to determine form. Morgan has shown that a definite amount of moisture is necessary for the development of the abnormal abdomen in *Drosophila*. Metz has several mutants that depend on specific cultural conditions for their recognition.

As shown by the constant results under constant external conditions, the hereditary mechanism remains the same. The reactions involved in differentiating the somatic tissues have been shown to be modifiable through various external factors, chief of which is temperature.

Consideration of the Means by Which Temperature Can Produce an Effect on Facet Number.

We have seen in the foregoing pages that facet number in the full-eyed wild fly is affected very little by temperature, while in the bar-eyed mutant there is a very marked effect. It is also evident that the effect is produced only through a relatively short period in larval life. What hypothesis can explain these facts?

Assuming a normal mechanism for facet production (A, B, C, D, E), we may say that temperature affects the rate of all the various processes involved in nearly equal amounts. The rates are increased at the higher temperatures, but at the same time the length of time of the reactions is proportionally shortened, thereby producing a constant number of facets (N) in the full eye.

In the bar eye a new condition has come about. The facet number is reduced to about $\frac{1}{4}$ that of the full eye at 15° and to about $\frac{1}{24}$ at 27° in the Low Selected line. In Ultra-bar the reduction is even greater; $\frac{1}{14}$ at 15° and $\frac{1}{40}$ at 27°.

Hypothesis 1. Reduction in Facet Number $\left(\frac{N}{a}\right)$ *Due to a Reduction in the Facet-Forming Substances* $\left(\frac{n}{a}\right)$.—As in the case of the full eye, increased rates at the higher temperatures with a decrease in the time of the reaction would produce a constant number of facets from a given amount of material at all temperatures.

Hypothesis 2. Surface Tension.—The number of facets in the bar-eyed stocks varies inversely with the temperature. The reaction thus has a negative temperature coefficient suggesting physical phenomena and particularly surface tension. The values of Q_{10} are far too high even to approximate the coefficients of any of the possible physical phenomena. Furthermore, if surface tension were the factor involved, we should expect it active in the full eye also, there producing similar temperature effects as in the bar.

Hypothesis 3. Reduction in the Amount of Facet-Producing Material Plus a Rate of Facet Production Independent of the Temperature.—In this case, the independent rate working through an increased time interval at the low temperatures would produce the greater number of facets as observed in the bar eye. However, when we apply the independent rate to full eye we should obtain a proportional difference at high and low temperatures, a condition shown not to exist.

Hypothesis 4. Considering the Reduction in Facet Number to be Due to an Inhibitor.—Assuming the inhibitor to be constant in amount for all temperatures, then if it follows the time-temperature laws of the other metabolic reactions, its rate will be decreased at the low temperatures, while the time during which it acts will be proportionally lengthened. At the higher temperatures the rate will increase while the time is proportionally shortened. Its action would therefore be constant and we would have the same number of facets at all temperatures.

Hypothesis 5. Considering the Reduction in Facet Number to Be Due to an Inhibitor, Constant in Amount, but with a Rate Independent of the Temperature.—Under these conditions the inhibitor working through an increased time interval at the lower temperatures would produce a greater reducing effect and we would find more facets at the higher temperatures.

Hypothesis 6. Considering the Amount of Inhibitor to Be a Function of the Temperature, and that More of It Is Produced at High Temperatures than at Low.—Obviously this condition would explain the results obtained in bar, but it is merely restating the question in another form, as we should then have to explain why more inhibitor was produced at one temperature than at another.

Hypothesis 7. Considering the Decrease in Facet Number to Be Due to an Inhibitor the Temperature of Which Coefficient Differs from That of the Normal Facet-Producing Reaction.—Let N be the normal number of facets in the full eye. Let nt be the length of the period at $T_1°$ during which facet rudiments are being produced. t is the length of the period at $T_2°$.

In full eye, at $T_1°$ N facets are formed at a rate of $\frac{N}{n}$ per t for a period of nt; at $T_2°$ N facets are formed at a rate of N per t for a period of t.

In bar eye, N is reduced to Bx at $T_1°$, to By at $T_2°$.

Facets are formed then at the rate of $\frac{Bx}{n}$ per t at $T_1°$.

Facet number is reduced at the rate of $\frac{N}{n} - \frac{Bx}{n}$ per t at $T_1°$.

Facets are produced at the rate of By per t at $T_2°$.

Facet number is reduced at the rate of N − By per t at $T_2°$. Then the rates of production and reduction have the following temperature coefficients.

$\frac{N}{n}$ = rate of production at $T_1°$

$Q_{T_2 - T_1} = n$

N = rate of production at $T_2°$

		When
$\frac{N - Bx}{n}$ = rate of reduction at $T_1°$	$Q_{T_2 - T_1} = n$	Bx = By
N − By = rate of reduction at $T_2°$	$Q_{T_2 - T_1} > n$	Bx > By
	$Q_{T_2 - T_1} < n$	Bx < By

In all the bar stocks, Bx is greater than By, where T_1 is the lower temperature. Hence the temperature coefficient for the reduction reaction in bar is greater than that of the production reaction. We can thus explain the difference in temperature relations between the full-eyed stocks and the bar-eyed stocks.

How can we explain the differences for the various bar-eyed stocks? From the above formulas it is obvious that the greater the difference between Bx and By the greater will be the difference between the Q_{10} for full and for bar. Bar is a changed condition which differs from the full in number of facets and also in the temperature coefficients of some of the reactions concerned in facet production. Is Ultra-bar a change in the same direction?

Ultra-bar effects a further decrease in the number of facets. But, as seen by the values of Bx and By, its temperature coefficient is really nearer that of the full than is that of the other bar stock. Ultra-bar is then not an increased condition of both these factors. The reversed change in the temperature coefficient may be a question of the concentration of the inhibitor.

Period during Which the Character of a Certain Structure Is Determined as Shown by the Temperature Effects.

Vernon[2] sums up the work of this nature on Lepidoptera. Dorfmeister concluded that the temperature had its greatest effect during the change from larva to pupa. According to Weismann, temperature acted at the beginning of the pupal period in *Vanessa prosa*. Merrifield concluded that the markings are chiefly affected during the early part of the pupal period, while coloring is affected during the penultimate pupal stage.

Hoge showed that exposure of the eggs to cold produced the greatest percentage of reduplication of legs in the imago of the fruit fly. Evidently the materials which determine the structure of the legs are differentiated in early embryonic development.

The bar eye factor comes into play after about three-fourths of the larval period is finished.

These last two cases are of interest in showing that some of the reactions which are involved in the differentiation of specific adult structures may occur at very early stages of immature life. Environmental stimuli must therefore be applied at definite periods in order to modify the organization of the adult.

The Direct Effect of Temperature upon the Mechanism of Inheritance.

Plough has shown that temperature has a definite effect on the percentage of crossing over between the hereditary materials of the second chromosome in *Drosophila*. He gets a maximum percentage at 13° and at 31° with a minimum from 22–27°. This curve Plough compares to the curve of Howell for the amount of contraction of the frog muscle at varying temperatures under constant stimulus. It is decidedly not a van't Hoff curve. Plough refers the phenomenon further to Lillie's results on the activation of the starfish eggs at various temperatures and concludes that temperature "probably causes some alteration in the physical basis of the egg."

The temperature effect on actual facet number seems to have nothing in common with the above results. If, however, we consider the per cent of increase or decrease per °C. some very interesting relations appear. Here, too, we find that the maximum change comes at the extreme high and low temperatures, with a minimum between, much as in the above reactions. With Plough, I leave the significance of the similarity of the cases to future research.

Individual Variation as Affected by Temperature.

In his book, Vernon[3] makes the statement that "variability becomes steadily greater as the environment becomes more unfavorable."

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[2] Vernon (1903), p. 241.

[3] Vernon (1903), p. 218.

In an earlier article (1895), he remarks that variability reaches a maximum at 18–20°, the temperature most favorable for development.

The temperature experiments on bar eye offer data on this subject. As was pointed out previously, the data are not altogether consistent when any attempt to draw striking conclusions is made.

An examination of the data published by Vernon is even less satisfactory and warrants neither of the conclusions above ascribed to him.

The present study has value only as a preliminary to the subject of the effect of temperature on individual variation. The two following conclusions are suggested if not proved. (1) When measured in terms of the coefficient of variability, variability increases with temperatures. (2) When measured in terms of standard deviation, variability decreases with increase in temperature.

Considerations of the Straight Line Feature of Physiological Reaction Curves, and of the Exponential Curve for Facet Number.

Variability in Q_{10}.

Variability in the temperature coefficient, Q_{10}, occurs in practically all chemical reactions. The typical variation is a slight decrease as the temperature rises. Trautz and Volkmann give some interesting values for saponification reactions in which there is first a slight increase and then a steady decrease in Q_{10} with increase in temperature.

The variation of Q_{10} for chemical reactions is in no way comparable to those of enzymatic and vital reactions.[4] In nearly every case the latter show a marked optimum. Obviously above the optimum Q_{10} becomes negative. As pointed out in this paper the values of Q_{10} for the rate of immature development vary from 27.31 at the 15–16° interval to − 2.41 at the 29–30° interval. These values are out of all proportion to the 2 to 3 requirements of van't Hoff's law.

This change in the value and sign of Q_{10} has been explained by Arrhenius and others as due to secondary factors. Two processes are involved; (1) the increase of activity of the enzyme, and (2) the destruction of the enzyme itself at the higher temperatures. The temperature, having a combative effect on the two processes, gives the appearance as it increases of checking the primary one when the end-results alone are considered.

Blackman accepts this sort of explanation for vital reactions. In the rate of assimilation by the leaves of the cherry laurel, he has ingeniously demonstrated the probability of the occurrence of increased rates above the optimum although these rates are not directly measurable.

Snyder has attributed the decrease in rate of physiological reactions at the higher temperatures to the differences in viscosity of protoplasm. This physical

[4] Ernst has shown an optimum in catalytic action of colloidal platinum upon H_2O_2.

phenomenon has a negative temperature coefficient. Experiments demonstrate a decrease in rate of nerve conduction with increase in viscosity at a constant temperature.

Balls maintains that the more rapid accumulation at higher temperatures of the waste products retards the primary reaction. While these products are formed at the lower temperatures, they are disposed of at a rate sufficient to prevent the checking of the primary reaction. At high temperatures they are formed more rapidly than they can be carried away. Their experimental removal, by dilution of the surrounding medium, raised the optimum of growth for the soreshin fungus considerably.

Coagulation of proteins, which has been advanced as an explanation of death at the higher temperatures, might be suspected of producing a retardation in rate at the submaximal temperatures.

Straight Line Physiological Reaction Curves.

Most physiologists have given up van't Hoff's formula as too inaccurate to have any practical value. They have abundantly demonstrated the metabolic rate relations to be a linear rather than an exponential function of the temperature.

Loeb's Hypothesis of Secondary Factors.

Loeb has recently explained the straight line character of the rate curves to be due to the flattening out of an exponential curve by secondary factors. He shows that the "rate of life" of the imago of *Drosophila* may be plotted as an exponential curve, and that there is no falling off at the higher temperatures.

The criticism can be made, however, that an examination of his rates above 31° demonstrates clearly the presence of secondary factors that would tend to convert a straight line curve into an exponential curve.

A consideration of these various explanations of straight line curves and optima is now in order in light of the present work.

Explanations of the Straight Line Temperature-Rate Relations and Optima, Based on the Data of Facet Number and Developmental Rate in Drosophila.

In the bar-eyed mutant of *Drosophila*, two distinct reactions have been examined in regard to the effect of temperature upon them. One gives a typical straight line curve with a marked optimum at 29°. The other gives an exponential curve without decrease in rate at the upper temperatures. From 15–27° these two curves approximate each other, suggesting a close similarity in the primary nature of the two reactions throughout. Above 27° these two curves diverge. Secondary factors have entered to retard the rate in one, and to transform an exponential curve into a straight line.

It is quite obvious that for the facet reaction there is no "enzyme destruction," as there is no falling off in rate at the higher temperatures. The optimum in the metamorphic curve shows that we are in the range of temperature where such destruction would be expected. The secondary factors then are not be to located in the principle of enzyme destruction.

It is likewise as evident that changes in viscosity of the protoplasm cannot explain the differences observed in the two reactions since both occur simultaneously in the same material.

We may extend the same objections to such explanations as coagulation of proteins, physical state of protoplasm, and allied phenomena.

Balls' explanation of optima consisted in the more rapid accumulation of waste products at high temperatures. The by-products of metabolism retard the rate of the primary vital reactions. Their experimental removal raised the optimum decidedly, but did not carry it to the maximum temperature of growth as would be the case were this the only explanation.

Differential Temperature Coefficients as an Explanation of the Straight Line Feature of Physiological Reaction Curves.

The one idea of Balls that shows greater possibilities of development is that of differential temperature coefficients. Vital reactions are a series of complex processes in which both chemical and physical phenomena are represented. It is inconceivable that all these should have the same temperature coefficients. Vernon (1895) has demon-

strated the fact in the gross anatomy of the echinoderm larvæ. Laughlin has recently shown that the various phases of mitosis have markedly different temperature coefficients. Osterhout has pointed out the complications arising from complex systems, in which the various reactions have different Q_{10} values.

Differentiation and growth are of a necessity synchronized processes. It is at the higher temperatures that the effects of diverse temperature coefficients would be most noticeable. If one stage in development must await another, it is quite obvious that the whole general process would be slackened in speed. At extreme temperatures regulation would become impossible.

As has been shown, the reaction by which the number of facets is determined is of relatively short duration. It is not complicated by the processes of growth. It shows a true chemical temperature coefficient throughout.

Metamorphosis involves many long and interdependent processes. The separate reactions do not have the same temperature coefficients. This is evident from Hertwig's curves for a close sequence of stages in the development of the frog tadpole. A rapid reaction must await with its end-products the slower one, before further development can proceed. The higher the temperature the more erratic will be the separate temperature effects, and the slower becomes the total rate of development.

It seems reasonable to conclude with Loeb that the straight line feature of physiological reaction curves, together with the special feature of optima, is due to the flattening out of an exponential curve by secondary factors. These factors are not specific such as enzyme destruction, viscosity changes, protein coagulation, or accumulation of waste products, but are the normal results of a differential temperature effect on the separate phases of growth, differentiation, and development.

The Inherited Effects of Temperature.

Induction.

Woltereck working on the size of the head in *Daphnia*, Middleton on the fission rate in *Stylonychia*, and Sumner on the length of the feet and tail in mice found that measurable effects could be produced by temperature. Furthermore the effect showed itself in a less degree in subsequent generations, although the

causal factor, extreme temperature, had been removed. To this phenomenon Woltereck gave the name of "induction" or "pre-induction" according to the number of generations involved.

No such effect as this was noted in connection with facet number as investigated in Experiment 51. It is possible, however, that by continued existence, generation after generation, at a high or low temperature, such an effect might be obtained.

Temperature as a Causal Factor in the Production of Mutations.

Tower found among his potato beetles color variations that persisted through subsequent generations. Presumably, since they were reared at high temperatures, the cause of the mutation lay in this fact.

The present study shows marked discontinuous variations at neither high nor low temperature. Temperature in the present study is not a factor in the production of mutations.

SUMMARY.

Three strains of the bar-eyed mutant of *Drosophila melanogaster* Meig have been reared at constant temperatures over a range of 15–31°C.

The mean facet number in the bar-eyed mutant varies inversely with the temperature at which the larvæ develop.

The temperature coefficient (Q_{10}) is of the same order as that for chemical reactions.

The facet-temperature relations may be plotted as an exponential curve for temperatures from 15–31°.

The rate of development of the immature stages gives a straight line temperature curve between 15 and 29°. Beyond 29° the rate decreases again with a further rise in temperature.

The facet curve may be readily superimposed on the development curve between 15 and 27°.

The straight line feature of the development curve is probably due to the flattening out of an exponential curve by secondary factors.

Since both the straight line and the exponential curve appear simultaneously in the same living material, it is impractical to locate

the secondary factors in enzyme destruction, differences in viscosity, or in the physical state of colloids.

Differential temperature coefficients for the various separate processes involved in development furnish the best basis for an explanation of the straight line feature of the curve representing the effect of temperature on the rate of physiological processes.

Facet number in the full-eyed wild stock is not affected by temperature to a marked degree.

The mean facet number for fifteen full-eyed females raised at 27° is 859.06.

The mean facet number for the Low Selected Bar females at 27° is 55.13; for the Ultra-bar females at 27° it is 21.27.

A consistent sexual difference appears in all the bar stocks, the females having fewer facets. This relation may be expressed by the sex coefficient, the average value of which is 0.791.

The average observed difference in mean facet number for a difference of 1°C. in the environment in which the flies developed is 3.09 for the Ultra-bar stock and 14.01 for the Low Selected stock.

The average proportional differences in the mean for a difference of 1°C. are 9.22 per cent for Ultra-bar, and 14.51 for Low Selected.

The differences in the number of facets per °C. are greatest at the low and least at the high temperatures.

The difference in the number of facets per °C. varies with the mean.

The proportional differences in the mean per °C. are greatest at the lower (15–17.5°) and higher (29–31°) temperatures and least at the intermediate temperatures.

Temperature is a factor in determining facet number only during a relatively short period in larval development.

This effective period, at 27°, comes between the end of the 3rd and the end of the 4th day.

At 15°, this period is initiated at the end of 8 days following a 1st day at 27°.

At 27° this period is approximately 18 hours long. At 15° it is approximately 72 hours long.

The number of facets and the length of the immature stage (egg-larval-pupal) appear related when the whole of development is passed at one temperature.

That the number of facets is not dependent upon the length of the immature stage is shown by experiments in which only a part of development was passed at one temperature and the remainder at another.

Temperature affects the reaction determining the number of facets in approximately the same way that it affects the other developmental reactions, hence the apparent correlation between facet number and the length of the immature stage.

Variability as expressed by the coefficient of variability has a tendency to increase with temperature. Standard deviation, on the other hand, appears to decrease with rise in temperature.

Neither inheritance nor induction effects are exhibited by this material.

This study shows that environment may markedly affect the somatic expression of one Mendelian factor (bar eye), while it has no visible influence on another (white eye).

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THE INFLUENCE OF HYDROGEN ION CONCENTRATION ON THE INACTIVATION OF PEPSIN SOLUTIONS.

By JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, February 14, 1920.)

One of the many factors which must be taken into consideration in any experiments with enzymes is the possible inactivation of the enzyme during the course of the reaction. This factor in the case of pepsin has been suggested by Sörensen[1] as the cause of the displacement of the optimum acidity for the digestion of protein to the acid side during the course of the digestion. He considers that the enzyme is more rapidly destroyed by the weak than by the strong acid. Arrhenius,[2] on the other hand, considers that the decrease in the rate of digestion on the acid side of the optimum hydrogen ion concentration for digestion is due to the more rapid destruction of the enzyme by the strong acid. If this explanation is correct the optimum phenomenon loses much of its significance and becomes a secondary characteristic of enzyme activity comparable to the optimum temperature. The possibility also arises that the peculiar falling off of the rate of digestion during the course of the reaction, at any hydrogen ion concentration, is also due to the destruction of the enzyme.

Several investigations[3] have been made on the stability of pepsin in acid solutions from various points of view but the results are not at all concordant. Much of this variation in results is probably due to the failure to realize the importance of the hydrogen ion concentration rather than the total acid concentration.

[1] Sörensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1909, viii, 162. Sörensen's experiments were made at 52°. They are therefore not strictly comparable with the present results.

[2] Arrhenius, S., Quantitative laws in biological chemistry, London, 1915, 44.

[3] Biernacki, E., *Z. Biol.*, 1891, xxviii, 49. Grober, J. A., *Arch. Exp. Path. u. Pharmacol.*, 1904, li, 103. Liebmann, P., and Johannesen, L., *Ugesk. Læger*, 1911, lxxiii, 902. Ramsay, C. F., *J. Am. Pharm. Assn.*, 1917, vi, 1047.

In the experiments considered in this paper the effect of the following variables on the inactivation of pepsin in solution has been studied: (1) the hydrogen ion concentration; (2) the anion of the acid; and (3) the purity of the enzyme solution.

The results of the experiments are given in Tables I and II and in Figs. 1 and 2. The figures in the tables are the relative amounts of active enzyme present in the solution after 24 or 48 hours. The total active enzyme present at the beginning of the experiment is taken as 10 units. The time required to cause a constant change

TABLE I.

Influence of the Purity of the Enzyme Solution on the Destruction of Pepsin at Various Hydrogen Ion Concentrations.

pH	Relative amount of pepsin per cc. of solution after 48 hrs. at 38°C.		
	0.25 per cent active pepsin.	2.5 per cent weak pepsin.	1.5 per cent weak pepsin in 3 per cent egg albumin solution.
6.2		1.0	
5.9			4.6
5.5	2.0	7.4	7.2
5.1	10.0		
4.7		8.4	8.2
4.0	10.0		7.9
3.6	9.0	6.8	7.2
2.0		6.3	7.0
1.2	8.0	6.0	6.8
0.6	7.8		

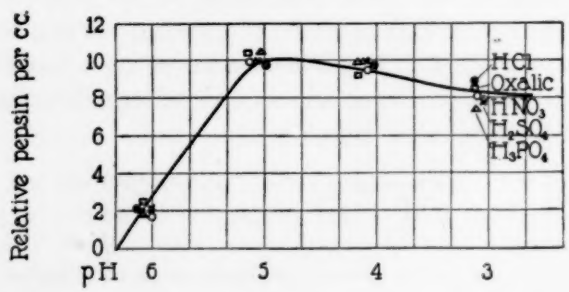

FIG. 1. Relative amount of active pepsin at different hydrogen ion concentrations with various acids after 24 hours at 38°C.

in the conductivity of an egg albumin solution under constant conditions is considered as inversely proportional to the amount of active enzyme present and was used as a measure of the enzyme concentration. The experiments with different enzyme solutions were not done at the same time and are not strictly comparable. The experiments with the various acids, however, are comparable.

It will be seen that in all the experiments the enzyme is most stable at a pH of about 5.0, irrespective of the anion of the acid and of the purity of the solution. Increasing the alkalinity of the solution causes a very great increase in the destruction of the enzyme. There is some indication that the impure solutions are inactivated more slowly under these conditions than the purer ones.

TABLE II.

Influence of Various Acids on the Destruction of Pepsin at Various Hydrogen Ion Concentrations.

pH	Relative amount of pepsin per cc. of solution containing the acids noted below after 24 hrs. at 38°C.				
	HNO_3	H_2SO_4	H_3PO_4	Oxalic.	HCl
6.0–6.2	1.7	2.1	1.8	2.5	2.2
5.0–5.2	10.0	10.0	10.3	10.0	9.8
4.0–4.2	9.5	10.0	10.0	9.2	9.6
3.0–3.2	8.0	7.9	7.6	8.5	8.8

Increasing the acidity of the solution above pH 5.0 causes a very slow increase in the amount of pepsin destroyed, and the quantity inactivated is not influenced either by the purity of the solution or by the anion of the acid. It would seem necessary to conclude from the marked asymmetry of the curve for the destruction of the enzyme, as plotted against the hydrogen ion concentration, that the process of inactivation of the enzyme on the acid side of pH 5.0 differs from the process of inactivation on the alkaline side of pH 5.0.

Fig. 2 shows that the amount of pepsin remaining in solution after 24 hours at 38°C. is about the same throughout the range of acidity in which the enzyme is active. The rate of destruction of the enzyme therefore differs very little at a pH of 1.0 and a pH of 3.0. As is well known, the activity of the enzyme varies greatly within this range.

This is shown by Curve D, Fig. 2, which is taken from Sörensen's paper and which represents the rate of digestion of egg albumin by pepsin at various hydrogen ion concentrations. If the decline in the rate of digestion on the acid side of pH 2.0 was due to the increased destruction of the pepsin by the acid in greater concentration than this, the same drop should be noticed in Curves A, B, and C as in Curve D, since these curves represent the actual amount of destruction of the enzyme at the acid concentration in question. Fig. 2 shows that this is not the case. But little more enzyme was destroyed at a

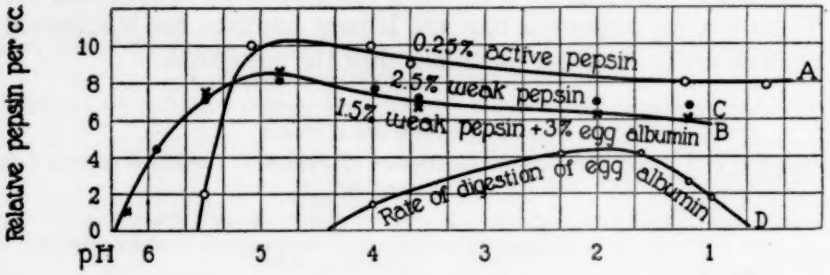

FIG. 2. Relative amount of active pepsin in various solutions at different hydrogen ion concentrations after 24 hours at 38°C.

pH of 1.0 than at a pH of 2.0 or 3.0. The rate of destruction is in any case much too slow to account for the rapid drop in the rate of the digestion curve. This drop is noticeable in the first few minutes of the reaction, while, as the figures show, only 10 to 20 per cent of the enzyme is destroyed in 48 hours at this hydrogen ion concentration.

The fact that the action of the acid on the enzyme is nearly the same across the whole range of hydrogen ion concentration in which the enzyme is active may be considered as indirect evidence that the optimum phenomenon is connected with changes in the substrate rather than in the enzyme. It is apparent from the figures that the enzyme is most stable at a pH of about 5.0; *i.e.*, the same as that for the isoelectric point of many proteins. There is no evidence, however, that pepsin is isoelectric at this point. A series of migration experiments made by the writer confirmed those of Michaelis and Davidsohn[4] (except that the enzyme was never found to migrate to both

[4] Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1910, xxviii, 1.

poles at the same pH) and gave a change in the direction of migration at about pH 3.0. There is no relation between this point and either the resistance of the enzyme to acid or the rate of its action on proteins. It is probable that this is not the isoelectric point of pepsin itself but that of a compound formed between pepsin and some other substance in the solution, since Peckelharing and Ringer[5] found that very pure pepsin solutions showed no isoelectric point.

No evidence was found that the inactivation of the enzyme was reversible under the conditions of these experiments although many experiments were made with this point in view.[6]

The results show that digestion experiments with pepsin cannot be carried out at 38° for longer than 24 hours without being complicated by the fact that the enzyme concentration is lower at the end of the experiment than at the beginning. They also show that in experiments on the decomposition temperature it is necessary to consider the reaction of the medium.

The general effect of the hydrogen ion concentration on the stability of the enzyme resembles that described by Falk[7] for lipase, and by Frankel[8] for papain. In the case of papain, however, the influence of the reaction is reversed; *i.e.*, papain is more sensitive to acid than to alkali.

Experimental Procedure.

Pepsin Preparations Used.—Active: Fairchild's pepsin U. S. P. 1:19,500. Weak: Pepsin U. S. P. 1:3,000.

Hydrogen Ion Determinations.—All determinations were made by the E. M. F. method.

Determination of the Relative Amount of Pepsin in Solution.

The enzyme solution was made up as shown in the tables and placed in a water bath at 38 ± 0.1°C. 5 cc. of the solution were pipetted out for analysis and 5 cc. of an acid solution added of such strength as to make the final acid concentration in each case equal

[5] Peckelharing, C. A., and Ringer, W. E., *Z. physiol. Chem.*, 1911, lxxv, 282.

[6] Tichomirow, N. P., *Z. physiol. Chem.*, 1908, lv, 107.

[7] Falk, K. G., *J. Biol. Chem.*, 1917, xxxi, 97.

[8] Frankel, E. M., *J. Biol. Chem.*, 1917, xxxi, 201.

to that of the solution containing the highest amount of acid. 1 cc. of this diluted solution was then added to a standard egg albumin solution and the time necessary to cause a 10 per cent change in the conductivity of the latter determined as described in a previous paper.[9] The relative concentration of active pepsin was about the same at the beginning of each experiment. This quantity was taken as 10 in each case. Under the conditions of these experiments neither the products of the digestion of the egg albumin nor the inactivated pepsin interferes with the determination; *i.e.*, the reciprocal of the time to cause a given change is directly proportional to the total quantity of active pepsin present.

SUMMARY.

1. Pepsin in solution at 38°C. is most stable at a hydrogen ion concentration of about 10^{-5} (pH 5.0).

2. Increasing the hydrogen ion concentration above pH 5.0 causes a slow increase in the rate of destruction of pepsin.

3. Decreasing the hydrogen ion concentration below pH 5.0 causes a very rapid increase in the rate of destruction of the enzyme.

4. Neither the purity of the enzyme solution nor the anion of the acid used has any marked effect on the rate of destruction or on the zone of hydrogen ion concentration in which the enzyme is most stable.

5. The existence of an optimum range of hydrogen ion concentration for the digestion of proteins by pepsin cannot be explained by the destruction of the enzyme by either too weak or too strong acid.

[9] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 113.

THE EFFECT OF THE CONCENTRATION OF ENZYME ON THE RATE OF DIGESTION OF PROTEINS BY PEPSIN.

By JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, February 16, 1920.)

The study of the kinetics of enzyme action has led almost invariably to results differing more or less from those predicted by the general laws of chemistry. It would be expected from the general theory of chemical reactions that enzyme reactions should conform to the law expressing the rate of a monomolecular reaction, accelerated by the presence of a catalyst. The rate of reaction, therefore, should be proportional to the concentration of the enzyme and of the substrate and should decrease with time as predicted by the monomolecular formula. This has been found to be true in rare instances[1,2]; but in general the monomolecular formula does not hold for enzyme reactions. It has been found in many cases that the products of reaction interfere with the action of the enzyme. This would account for the divergence of the rate of reaction from that predicted by the monomolecular formula, since, owing to the action of the products, the concentration of the enzyme is changing during the course of the reaction, while the monomolecular formula takes account only of the changes in concentration of the substance decomposed. The rate of reaction of two solutions containing different amounts of enzyme, however, if compared during the same stage of the reaction, should be porportional to the quantity of enzyme, since any effect of the products should be the same in both solutions. It is found in many instances that this is not the case. Enzyme reactions diverge from the expected course of such reactions not only as regards the change in rate with the progress of the reaction, but also in regard to the relation between the rate and the concentration of substrate or enzyme.

[1] Euler, H., *Z. physiol. Chem.*, 1907, li, 213.

[2] Taylor, A. E., *J. Biol. Chem.*, 1906–07, ii, 87. Also Schmitz, H., *J. Gen. Physiol.*, 1919–20, ii, in press.

It was suggested by Brown[3] that these divergences in the case of invertase were due to the fact that the enzyme formed an intermediate compound with the substrate; and several formulas[4] which fit the experimental facts fairly well have been derived on this assumption. They all contain several arbitrary constants, however, and in the lack of any direct evidence in favor of the mechanism which they assume the agreement between calculated and observed values can hardly be considered conclusive. It is assumed in attempting to explain the mechanism of enzyme reactions from the point of view outlined above, that all the enzyme and all the substrate molecules present are equally able to take part in the reaction; in other words, that the active concentration and total concentration of enzyme (or substrate) are the same or directly porportional to each other. It is obvious that, if the active concentration of substrate or enzyme was not equal to the total concentration, the law of mass action would fail to hold if the total concentrations were used in formulas derived from this law, since the law itself states only that the rate of reaction is proportional to the active concentration of the reacting substances. It appears *a priori* quite possible that active enzyme or substrate molecules may exist in solution in equilibrium with other molecules which do not take part in the reaction. The concentration of active enzyme molecules (in the sense of the law of mass action, *i.e.* those which take part in the reaction) would then be some other function of the total concentration and would not be directly proportional to it. The rate of reaction would then also be found to vary as some other function of the total enzyme concentration and not in direct proportion to it. An exactly analogous case is well known in general chemistry; namely, acid hydrolysis.[5] The hydrogen ion is the active part

[3] Brown, A. J., *J. Chem. Soc.*, 1902, lxxxi, 373.

[4] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 146. These authors review the various other formulas proposed. See also Moore, B., in Hill, L., Recent advances in physiology and biochemistry, New York and London, 1906, 43.

[5] For a general discussion of this question see Stieglitz, J., and collaborators, *Am. Chem. J.*, 1908, xxxix, 29, 166, 402, 650. Stieglitz's experiments were made on the hydrolysis of esters. These solutions can hardly be considered heterogeneous and yet show the same divergences from the simple mass action law as do enzyme reactions. This question will be discussed more fully in a subsequent paper.

of the molecule and the rate of reaction therefore varies directly with the hydrogen ion concentration and not with the total acid concentration. In sufficiently dilute solutions the two of course become practically identical since the acid is then completely dissociated. It will be shown in the succeeding part of this paper that pepsin solutions obey the same laws as weak acid solutions in regard to the relation between the total concentration and the rate of hydrolysis; and that the divergence from the law of mass action is not due to any peculiarity of the enzyme reaction itself, but to the fact that the active enzyme concentration is not always directly proportional to the total enzyme concentration.

Experimental Procedure and Results of the Present Investigation.

In a former paper[6] a method was described for determining the rate of pepsin digestion by means of changes in the conductivity of an egg albumin solution to which the pepsin had been added. From these results the time necessary to cause a given change in the conductivity of the solution was determined by graphic interpolation. In the experiments reported in this paper the time in hours necessary to cause the first 10 per cent change was taken as the standard. The reciprocal of this time then $\left(\frac{1}{T \text{ hours}}\right)$ is proportional to the mean rate of digestion for the first 10 per cent of the reaction. For convenience this value will be spoken of as the amount of "active pepsin." The volume noted in the tables is considered in every case as the number of cc. of diluted enzyme solution containing 1 cc. of the original enzyme solution. It is therefore a measure of the dilution of the pepsin before adding to the egg albumin solution. Since 1 cc. of this diluted solution was added to 25 cc. of egg albumin in order to make a determination, the concentration of the pepsin during the actual digestion was $\frac{1}{26}$ of that shown in Tables I, II, III, and V. The conductivity and pH of all solutions were kept equal as nearly as possible. It was pointed out that this change in conductivity did not exactly parallel the change in amino nitrogen of the solution, and so cannot be considered as representing the true course

[6] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 113.

of the reaction. If the amount of egg albumin and all other factors except the amount of pepsin are kept equal, however, the time necessary to cause a given change may be considered as a definite measure of the rate of reaction, which is all that is necessary for the present purpose.

It was stated[6] that the rate of reaction (*i.e.* the reciprocal of the time to cause a given change) was directly proportional to the concentration of enzyme solution, and that any products of reaction present in the enzyme solution did not interfere with the reaction. Both statements were true as regards the pepsin solutions used in the experiments reported. It was found, however, that some pepsin solutions did not obey this law. The rate of digestion, instead of being directly proportional to the enzyme concentration, increased much more slowly. The same phenomena have been observed by Bayliss in the case of trypsin[7] and invertase,[8] and have frequently been observed in enzyme reactions. It has formed one of the arguments for the conception that the enzyme combines with the substrate according to the adsorption formula.[8,9]

Table I is a summary of an experiment illustrating this point. The results are shown graphically in Fig. 1. It is obvious that the value of ET (total pepsin concentration $\times$ the time necessary to cause 10 per cent of the total change in conductivity) is constant for low concentrations but increases in higher concentrations. (If the rate of reaction is directly proportional to the enzyme concentration, the value of ET must of course be constant.) The calculated figures were obtained by a formula considered below. The key to this behavior is given by the results of the experiments shown in Table II. In this experiment 2.5 cc. of an active pepsin preparation were diluted to 10 cc., A, with HCL (pH 2.0) and, B, with a solution of "peptone"[10] prepared by the digestion of egg albumin by a very small amount of pepsin (but containing no active pepsin). Solutions A and B were

[7] Bayliss, W. M., *Arch. Sc. Biol.*, 1904, ii, suppl., 261.

[8] Bayliss, W. M., *Proc. Roy. Soc. London, Series B*, 1911–12, lxxxiv, 90. Duclaux, E., Chimie Biologique, Paris, 1883.

[9] Bayliss, W. M., The nature of enzyme action, Monograph on Biochemistry, London, New York, Bombay, and Calcutta, 3rd edition, 1914.

[10] The word peptone is used in this paper as a general term for substances with which pepsin combines in solution, but does not hydrolyze.

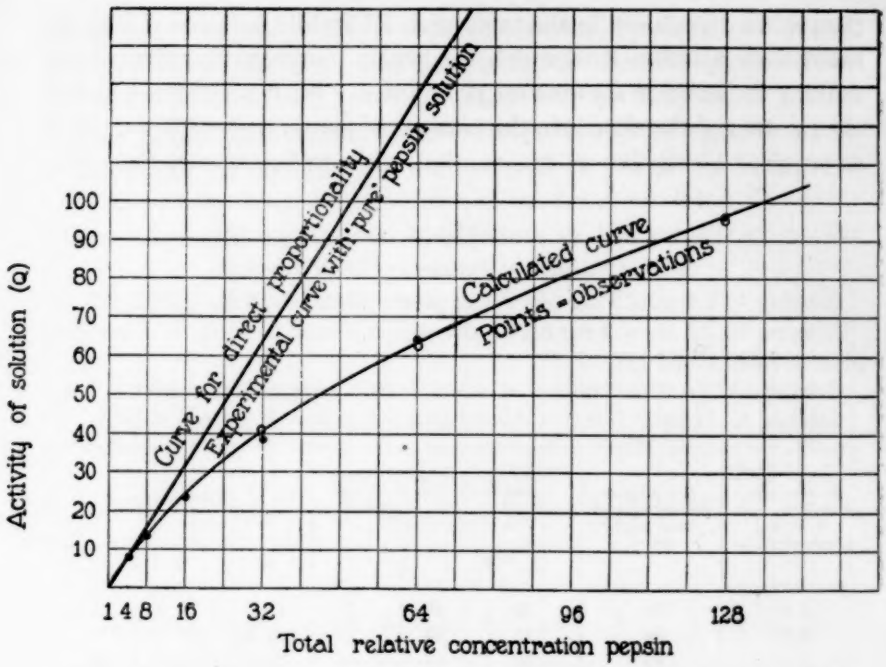

FIG. 1. Curves showing pepsin concentration and rate of digestion (*cf.* Table I).

TABLE I.

Enzyme Concentration and Rate of Digestion.

Pepsin solution. 10 per cent solution of Grübler's pepsin in HCl, pH 2.0.

$$K = 7.2 \qquad d = \frac{30}{v}$$

V = volume containing 1 cc. of original pepsin solution.	E = total pepsin per cc.	$Q = \frac{1}{T}$ = active pepsin per cc.					ET
		Observed.				Calculated.	
		1	2	3	Average.		
1	26.9	9.1	9.7	10.0	9.6	9.7	269
2	13.44	6.25	6.30	6.67	6.39	6.40	206
4	6.72	4.17	3.70	3.57	3.81	4.05	175
8	3.36	2.38	2.50	2.17	2.35	2.42	145
16	1.68	1.39	1.43	1.35	1.39	1.38	120
32	0.84	0.83	0.80	0.78	0.80	0.77	106
64	0.42	0.41	0.40	0.39	0.40	0.40	100
128	[0.21]	0.22	0.20	0.20	0.21	0.20	100

then diluted as shown in the table with HCl while Solution C was diluted with Solution B in which the pepsin had been inactivated by making the solution alkaline for 10 minutes. With Solutions A and C the product of the time into the amount of pepsin present is constant as required by the law of mass action, while in Solution B the value

TABLE II.

Effect of Addition of Peptone to Pepsin Solutions.

Solution A. 2.5 cc. of 2 per cent active pepsin diluted to 10 cc. + HCl, pH 2.0.

Solution B. 2.5 cc. of 2 per cent active pepsin diluted to 10 cc. + 1 per cent peptone solution, pH 2.0.

Solutions A and B then diluted as noted + HCl. Pepsin determined in 1 cc.

Solution C. Same as B except diluted with inactivated B, instead of HCl.

V = volume containing 1 cc. of original pepsin solution.	E = relative concentration of total pepsin taken.	Time for 10 per cent change in conductivity of 25 cc. of egg albumin + 1 cc. of solution. T = hrs. × (10^3).			ET for solution.		
		A	B	C	A	B	C
1.0	100	20	28	21	20.0	28.0	21.0
1.5	66	31	34	29	20.5	22.5	19.1
2.0	50	40	39	41	20.0	19.5	20.5
4.0	25	83	79	81	20.7	19.8	20.3
8.0	12.5	170	162	178	21.2	20.3	22.2

of the product decreases with increasing dilution until it becomes equal to that value obtained from Solutions A and B, and then remains constant.[11] The results are plotted in Fig. 2. The straight line represents direct proportionality.

The solutions were made up to contain the same total concentration of pepsin and in the higher dilutions show the same degree of activity. It seems, therefore, that the divergence of Solution B from the regular law must be due to the fact that the peptone combines with the pep-

[11] This experiment is probably the explanation of the conflicting results obtained by Bayliss[8] and Nelson and Vosburgh (Nelson, J. M., and Vosburgh, W. C., *J. Am. Chem. Soc.*, 1917, xxxix, 790) in connection with the action of invertase. The activity of the solution of invertase used by Bayliss was not proportional to its concentration whereas the activity of that used by Nelson and Vosburgh was directly proportional.

sin to form a rather highly dissociated compound and that the pepsin so combined is inactive. The concentration of active pepsin would therefore be decreased by the peptone and the decrease would be greater in concentrated than in dilute solution. This hypothesis also accounts for the results of Experiment C in which the solution is diluted with an inactivated portion of the same solution. If the inactivated pepsin enters into equilibrium in the same way as the

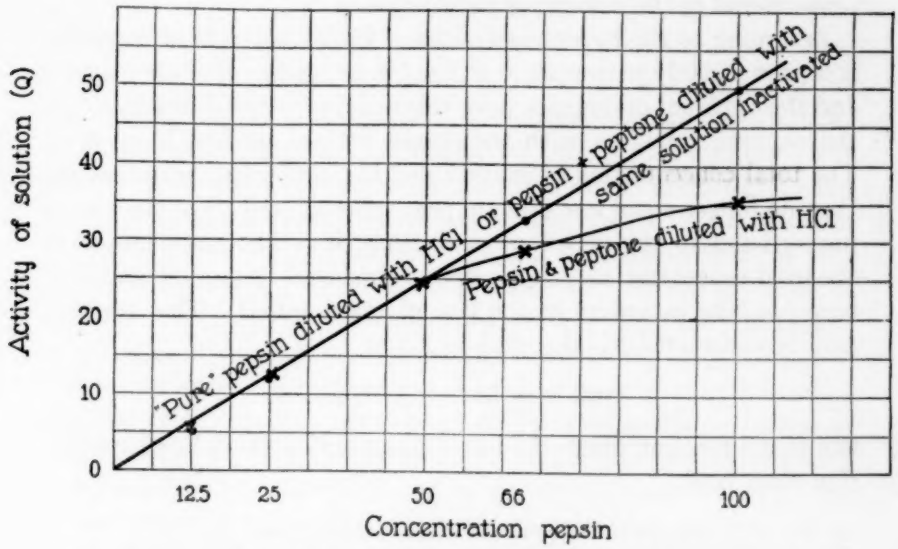

FIG. 2. Curves showing effect of peptone on activity of diluted pepsin solutions (*cf.* Table II).

active pepsin, the concentration of active pepsin in a solution, diluted with an inactivated portion, should decrease in direct proportion to the total concentration. The experiment shows that this is the case. (This question will be taken up more fully later.) The results of this experiment show also that in order to determine the total amount of pepsin present in solution it is necessary to use a dilution such that the rate of digestion is directly proportional to the amount of enzyme solution taken. If this is done the value for the total amount of enzyme, found at dilutions where this value has become constant, is an experimental determination of the total amount of enzyme present, expressed, however, in arbitrary units.

The effect of the peptone in Solution B might be qualitatively explained by the hypothesis that the peptone in the solution combines with the substrate and so reduces the concentration of active substrate molecules, thereby causing the enzyme to become "saturated" with substrate. This explanation, however, fails to explain the results of Experiment C since the same concentration of peptone is present here as in Solution B and yet in this experiment the rate *is* proportional to the amount of enzyme taken.

According to the hypotheses outlined above, the rate of digestion is always directly proportional to the concentration of active pepsin; and the apparent divergence from this relation is due to the fact that the peptone combines with the pepsin and so renders it inactive. The total concentration of enzyme and the active concentration are then no longer equal nor directly proportional; and since the rate is proportional to the active concentration, it is not proportional to the total concentration. It is also assumed that the pepsin and peptone combine according to the law of mass action. This reaction may be considered to take place as follows:

$$\text{Pepsin} + \text{peptone} \rightleftharpoons \text{pepsin}-\text{peptone}$$

and if the reaction obeys the law of mass action the following equation must hold.

$$\frac{\text{Concentration pepsin} \times \text{concentration peptone}}{\text{Concentration pepsin-peptone}} = K \quad (1)$$

or

$$\frac{Q \cdot (d - (E - Q) + x)}{E - Q} = K \quad (2)$$

where E is the total enzyme concentration, Q is the concentration of active (uncombined) pepsin, d is the concentration of peptone present at the beginning of the reaction, and x is the amount of peptone formed during the course of the reaction at the time t. K is the equilibrium constant expressed in arbitrary units since it contains the unit of measurement used. (For the sake of simplicity only the case is considered in which the substance combined with the pepsin at the beginning of the reaction is the same as that formed during the

digestion.) The value of Q then (the active pepsin concentration) at any moment of the reaction would be that defined by equation (2) or

$$Q = -\frac{d - E + x + K}{2} + \sqrt{\left(\frac{d - E + x + K}{2}\right)^2 + KE} \quad (3)$$

The differential equation for the whole process[12] would then be

$$\frac{dx}{dt} = kQ(A - x) \quad (4)$$

in which Q has the value expressed in equation (3), and A is the active concentration of substrate present at the beginning of the reaction. (It seems quite probable that the active substrate concentration is related to the total substrate concentration in the same way as the active and total enzyme concentrations are related. This question will be discussed later. For the present it is assumed that the rate is proportional to the substrate concentration. At low dilutions of substrate this is an experimental fact.) If this value for Q is substituted in equation (4) it becomes too unwieldy in the integral form to use conveniently. The equation may be tested in the differential form, however, by choosing a small constant value for Δx (taken as 10 per cent of the total change in these experiments) and determining Δt experimentally. The reciprocal of the time necessary to cause the change will then be proportional to the mean rate of reaction during the first 10 per cent of the hydrolysis. This rate is of course decreasing constantly due (1) to the decrease in substrate concentration, and (2) to the decrease in the concentration of active pepsin since some pepsin is removed by combination with the products of reaction. The relative decrease in Q due to (1) is the same in every case and cancels out in comparative experiments, such as are considered here, since the total substrate concentration is kept the same in every experiment. The relative decrease in rate (Q) due to (2), however (as may be seen from equation (2)), will not always be the same but will depend to some extent on the relative values of E, d, and x. It will be shown later that a 5 per cent egg albumin solution when completely digested contains about 10 units of peptone (Table III). The

[12] Neglecting any effect of the reverse reaction.

value of x therefore in the first 10 per cent of the reaction will increase from 0 to 1.0. The percentage decrease in Q (the concentration of active pepsin) will depend to some extent on the concentration of peptone (d) present at the beginning of the reaction. That this is actually so is shown by the fact that the relative rates of digestion of two solutions containing the same amount of pepsin but very

TABLE III.

Enzyme Concentration and Rate of Digestion.

Pepsin solution. 1 per cent active pepsin + 10 per cent egg albumin, pH 2.0. Digested 24 hrs. at 37°C. Diluted as below + HCl, pH 2.0.

$$K = 8.5 \qquad d = \frac{19.2}{v}$$

V = volume containing 1 cc. of pepsin solution.	E = total pepsin.	$Q = \frac{1}{T}$ = active pepsin.			
		Observed.			Calculated.
		1	2	Average.	
1.0	10.95	4.35	4.24	4.29	4.40
1.18	9.32	4.17	4.05	4.11	4.08
1.43	7.66	3.84	3.57	3.70	3.71
1.66	6.58	3.45	3.50	3.47	3.39
2.0	5.48	3.03	2.98	3.00	3.06
2.5	4.39	2.94	2.78	2.86	2.67
3.33	3.28	2.17	2.38	2.27	2.17
5.0	2.18	1.75	1.50	1.62	1.61
10.0	1.09	1.0	0.98	0.99	1.05
20.0	0.54	0.57	0.52	0.55	0.52

different amounts of peptone vary, depending on what stage of the reaction is compared. This is due to the fact that the rate of digestion of the solution containing the peptone decreases more slowly than that of the solution containing no peptone. This is in agreement with the formula. The differences in the percentage decrease in the rates of digestion of two solutions during the first 10 per cent hydrolysis, due to variations in the relative values of E and d, were found to be too small to effect the results within the range of values of E and d used in these experiments. The relative mean rate for the first 10 per cent hydrolysis may therefore be considered proportional

to the amount of active pepsin present at the beginning of the reaction; *i.e.*,

$$\text{Rate} = \frac{1}{T} = Q = -\frac{d-E+K}{2} + \sqrt{\left(\frac{d-E+K}{2}\right)^2 + KE}$$

where T is the time in hours necessary to complete the first 10 per cent of digestion. This equation may be tested experimentally by testing the constancy of K for various values of E and d or, better, by comparing calculated and observed values of $\frac{1}{T}$ since small experimental errors cause very large changes in the value of K.

The results of such a series of experiments have been given in Table I. Table III contains the results of a similar experiment in which the pepsin solution was prepared by adding 10 per cent of egg albumin to an active pepsin solution and allowing digestion to be completed at a temperature of 38°C. The solution was then diluted as shown in Table III. As was the case in Experiment 1, the rate of digestion is not directly proportional to the total enzyme concentration. It will be seen that in both Tables I and III the agreement between calculated and observed values is within the experimental error. The figure for E, the total enzyme present, is determined directly from the experiments in high dilution when the value of ET has become constant. It was shown in Experiment 2 that the value for E obtained in this way was really proportional to the total amount of enzyme present. The value for d, the amount of peptone present at the beginning of the reaction, is determined from the figures themselves and therefore must be considered as a second arbitrary constant. This fact, of course, detracts considerably from the significance to be attached to the agreement between the observed and calculated values. It will be shown below, however, that under certain conditions the formula may be still further simplified so as to contain one constant and that it is still found to hold.

Table IV contains a summary of an experiment in which the total concentration of peptone was kept the same and the concentration of pepsin increased. The results are shown graphically in Fig. 3. The values for E, the total pepsin present in the solution of Grübler's pepsin, K, the equilibrium constant, and d, the concentration of pep-

tone originally present, were taken from Table I. The value for E in Solution B was determined by a separate experiment. It will be seen that the total amount of active pepsin found in the solution is not equal to the sum of the amount of active pepsin added plus the amount of active pepsin already present. This shows that the pepsin

TABLE IV.

Addition of Active Pepsin Solution to Solution of Grübler's Pepsin.

Solution A. 10 per cent Grübler's pepsin, pH 2.0.

Solution B. 3 per cent active pepsin, $E = 4.2$.

$K = 7.2$, $d = 3.0$, E (in Solution A) = 2.69 (Table I). 1 cc. of Solution A + noted cc. of B made up to 10 cc. $K = 7.2$.

Volume of Solution B added.	Units of active pepsin added per cc.	Units of active pepsin in Solution A per cc.	$Q = \frac{1}{T}$ = total units of active pepsin.	
			Found.	Calculated.
cc.				
0	0	[2.08]	2.08	2.03
1	0.42	2.08	2.32	2.27
2	0.84	2.08	2.83	2.71
3	1.26	2.08	3.12	3.05
5	2.10	2.08	3.84	3.77
7	2.94	2.08	4.44	4.50
9	3.78	2.08	5.20	5.26

is in equilibrium with the substance that inhibits its action. The fact that the calculated values agree with those found by experiment shows that the equilibrium obeys the law of mass action since the calculated figures are obtained by means of this law.

The Effect of Inactivated Pepsin on the Equilibrium.

The results of Experiment 2 show that if a solution of pepsin (A) containing peptone is diluted with acid, the activity of the resulting solution *is not* directly proportional to the concentration of A. If the same solution is diluted with a portion of itself in which the pepsin has been inactivated with alkali, the activity of the resulting solution *is* directly proportional to the concentration of A. This is the result predicted if it is assumed that the inactive pepsin enters into the equilibrium (*i.e.* combines with the peptone) to the same extent as

the active pepsin. Table V summarizes the result of an experiment similar to Experiment 2 but covering a wider range. In this experiment an impure solution of pepsin (the same as used in Experiment 1) was diluted, A, with acid of the same hydrogen ion concentration, and, B, with the same solution which had been previously inactivated by alkali and then brought back to the same pH as the original. The

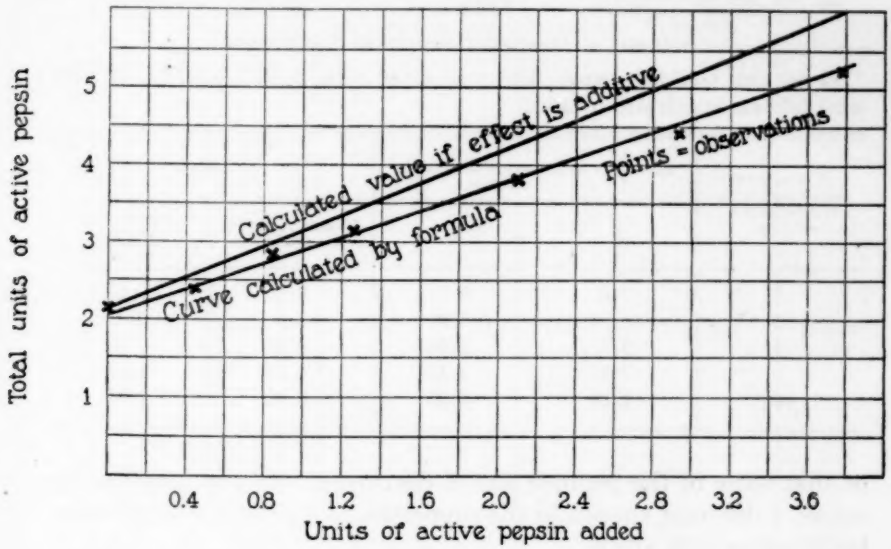

FIG. 3. Curves showing effect of addition of "pure" pepsin to pepsin solution containing peptone (*cf.* Table IV).

activity of the solution diluted with acid is not directly proportional to its concentration. When the same solution is diluted with an inactivated portion of itself, the activity of the resultant solution is directly proportional to its concentration. The figures show again that the result is predicted quantitatively by the hypothesis. Pepsin inactivated by alkali therefore retains the ability to combine with peptone exactly as does the active pepsin. It has, however, lost the power to hydrolyze protein. A very similar phenomenon is known in immunology—the so called toxoids; *i.e.*, toxins which are no longer injurious but are able to bind antibody in the same way as true toxin.

If the pepsin is inactivated by boiling instead of by treatment with alkali the results become irregular and do not agree with the hypothesis that the inactivated pepsin either does or does not enter into the equilibrium.[13] In order to predict them quantitatively it becomes necessary to assume that either the equilibrium constant is changed

TABLE V.

Influence of Inactivated Pepsin on Equilibrium.

10 per cent Grübler's pepsin diluted as noted with, A, HCl, pH 2.0. B, with same solution inactivated by alkali.

V = volume containing 1 cc. of original pepsin solution.	$Q = \frac{1}{T}$ = active pepsin observed in solution.		Calculated.	
	A	B	If inactive pepsin enters equilibrium.	If inactive pepsin does not enter equilibrium.
1	9.6	9.5	9.6	9.6
2	6.39	5.0	4.8	3.6
4	3.81	2.48	2.40	1.4
8	2.35	1.33	1.20	0.8
16	1.39	0.70	0.60	0.4

or that some of the peptone also is destroyed. In any case boiling causes a different change in the properties of a pepsin solution from inactivation with alkali.

Effect of Adding Increasing Amounts of Peptone to Pepsin Solutions.

It is possible to test further the hypothesis outlined in this paper by noting the effect of adding different amounts of peptone to a constant quantity of pepsin and comparing the observed and calculated activity of the resultant solution. If, as assumed in the hypothesis, the pepsin combines with the peptone to form a dissociated compound, the effect of adding successive equal amounts of peptone to a constant quantity of pepsin should not result in a constant decrease in activity of the solution for each unit of peptone added. The first unit of

[13] An apparently similar phenomenon was noticed by Bayliss[7] in his experiments with trypsin.

TABLE VI.

Effect of Adding Increasing Amounts of Peptone to Pepsin Solution.

0.5 cc. of 5 per cent active pepsin solution + noted cc. of peptone solution (from digested egg albumin) and made up to 10 cc. K = 8.5 (Table III), E = 3.16, d = 0.85 per cc. of peptone solution.

Peptone solution.	d = units of peptone added.	$Q = \frac{1}{T}$ = units of active pepsin.			Units of combined pepsin per unit of peptone added. Observed.	Qd
		Observed.	Average.	Calculated.		
cc.						
0	0	3.03 3.14 3.33	3.16			
1	0.85	2.86 2.86 2.94	2.88	2.92	0.33	2.4
2	1.7	2.70 2.70 2.86	2.75	2.75	0.24	4.6
4	3.4	2.38 2.40 2.50	2.42	2.42	0.22	8.2
6	5.1	2.08 2.04 1.96	2.03	2.13	0.22	10.0
8	6.8	1.67 1.85 1.92	1.81	1.91	0.20	12.0

peptone added should have a greater effect than the second, the second a greater than the third, and so on; the relative decrease of the effect depending on the value of the equilibrium constant. Table VI and Fig. 4 give the result of an experiment carried out in this way. It will be seen that the effect of adding increasing units of peptone agrees very well with the calculated values. The compound pepsin-peptone is widely dissociated at this dilution inasmuch as with a total concentration of 0.85 units of peptone and 3.16 units of pepsin only 0.28 units are combined. This fact is shown graphically in

Fig. 4 where the straight line represents the concentration of active pepsin which would be present if the combination was complete. Table VI also shows that the first unit of peptone inactivates more pepsin than the second, etc. This phenomenon is also common in immunology and is known as Ehrlich's phenomenon. As Arrhenius[14] has pointed out it is a general property of any equilibrium system.

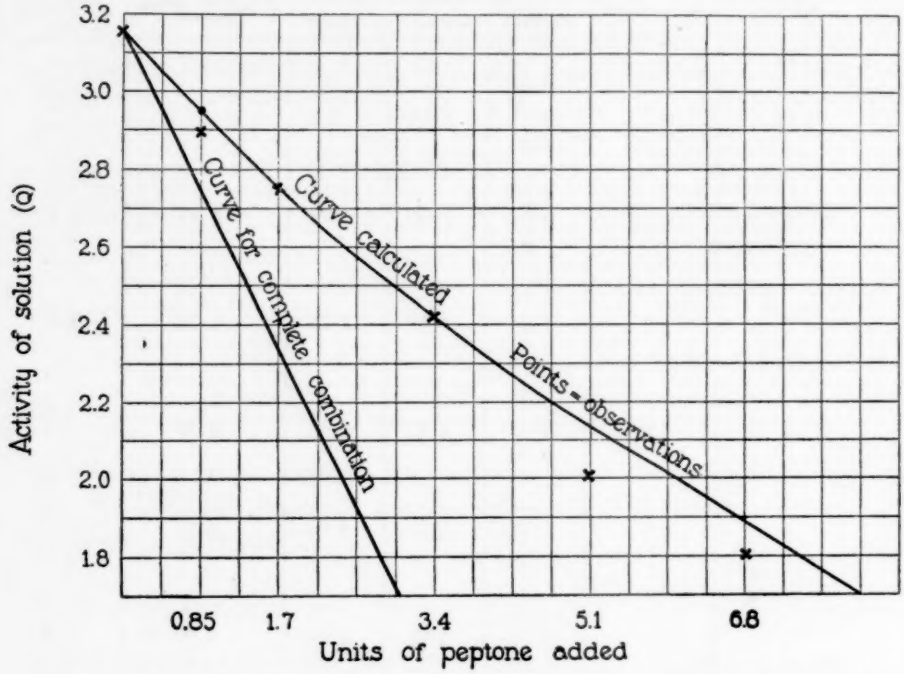

FIG. 4. Curves showing effect of adding increasing amounts of peptone to pepsin solutions (*cf.* Table VI).

In several other respects the action of pepsin on an egg albumin solution is more or less analogous to the action of toxin on an organism. In a sense the pepsin may be said to make the egg albumin solution immune to pepsin. That is, if a small amount of pepsin is allowed to act for a long time on a large quantity of albumin it will at first digest it very rapidly and the rate of digestion will be proportional to the amount of pepsin added. The rate of digestion decreases rapidly,

[14] Arrhenius, S., *Ergebn. Physiol.*, 1908, vii, 480.

however, and finally becomes almost negligible in spite of the fact that there is still a large amount of egg albumin in solution and that the pepsin still retains its activity (as may be demonstrated by diluting the solution, after which digestion will continue). The addition of a further amount of pepsin to the solution will now have little or no effect. The albumin solution is "immune" to the pepsin. This is due to the fact that a small amount of pepsin can cause the production of a very large amount of peptone. Each unit of peptone produced decreases the amount of free pepsin somewhat; but as may be seen from equation (2) it would require an infinite concentration of peptone (d) to reduce the concentration of free pepsin (Q) to 0. Practically, the reaction stops owing to the destruction of the pepsin.[15]

Referring again to Table VI, it will be noted that Qd, the product of the concentration of active enzyme into the concentration of peptone, approaches a constant value as d increases. In other words the concentration of active enzyme becomes nearly inversely proportional to the concentration of peptone, when the latter is present in great excess. This is a well known property of mass action equilibria and follows from the formula, as may be seen below. The formula used in this connection is

$$\frac{Q \cdot [d - (E - Q)]}{E - Q} = K$$

where Q is the concentration of active (free) enzyme, $[d-(E-Q)]$ the concentration of free peptone, and $E-Q$ the concentration of combined pepsin or peptone. It is obvious that as d increases Q must decrease so that the value of the term $E-Q$ approaches the constant value E. When d becomes very large compared with E the term $d-(E-Q)$ will not differ significantly from d. The equation may then be written

$$Q = \frac{KE}{d}$$

d in this equation represents the amount of peptone present at the beginning of the reaction. If the equation is to hold throughout the reaction the concentration of peptone will be represented by $d+x$.

[15] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 465.

If the simplest case is considered in which there is no peptone present at the beginning of the reaction the concentration of peptone at any time is x (since d is 0) and the formula becomes

$$Q = \frac{KE}{x}$$

This value for Q may now be substituted in equation (4) which becomes

$$\frac{dx}{dt} = \frac{KE(A - x)}{x} \quad (5)$$

in which A is the concentration of substrate at the beginning of the reaction and x is the amount of substrate decomposed (or of peptone formed) at the time T. For the first part of the reaction the value of $(A - x)$ will not differ much from the value of A and the equation may be still further simplified to

$$\frac{dx}{dt} = \frac{KEA}{x}$$

which states that the rate of digestion at any moment is directly proportional to the enzyme concentration and the substrate concentration, and inversely proportional to the amount of substrate decomposed. K in this equation is a new constant equal to the product of k, the velocity constant, and K (equation (4)), the equilibrium constant. This equation, as has been pointed out by Arrhenius,[16] is the differential form of Schütz's[17] rule, since on integration it becomes

$$TKEA = x^2 \quad \text{or} \quad x = K\sqrt{TEA}$$

That is x, the quantity of peptone formed, is proportional to the square root of the time, the concentration of pepsin, and the con-

[16] Arrhenius, S., *Medd. Kong. vetsakad. Nobelinst.*, 1908, i. An equation similar to this but containing $x\frac{1}{2}$ was found by Bodenstein and Fink (Bodenstein, M., and Fink, C. G., *Z. physik. Chem.*, 1907, lx, 1) to represent the rate of oxidation of SO_2 in the presence of platinum. Dernby, K. G., *Z. physiol. Chem.*, 1914, lxxxix, 425.

[17] Schütz, E., *Z. physiol. Chem.*, 1885, ix, 577.

centration of substrate. It follows from this that if two solutions are compared, each containing the same quantity of substrate, and allowed to digest the same length of time, but with varying concentrations of enzyme, the amount of substrate digested will be proportional to the square root of the enzyme concentration. This is the usual form of Schütz's rule.

It will be remembered that in the derivation of this equation two simplifying assumptions were made: (1) that x, the concentration of peptone, is large compared to Q, the concentration of active pepsin; and (2) that the quantity of substrate present remains relatively constant. The first condition is fulfilled as soon as the digestion has progressed more than a few per cent, provided the original concentration of pepsin is small compared to the concentration of albumin. The second condition, on the other hand, fails to hold after more than 30 or 40 per cent of the substrate is digested. It can be predicted then that Schütz's rule will not hold during the first few minutes of the reaction, or at the end of the reaction, or if the enzyme concentration is too high. As is well known, this is exactly the result obtained by experiment (*cf.* Arrhenius[16]).

The failure of the rule to hold during the first part of the digestion is due to the fact that x at this time is not large compared with Q and hence the relative change in Q is not inversely proportional to the change in x (as assumed in the derivation of the equation) but is much slower as demanded by equation (2). In order to express the fact correctly for the first part of the reaction, then, it would be necessary to substitute for Q in equation (4) the value of Q as defined by equation (3). As has been previously stated, this expression is too unwieldy to handle conveniently. The discrepancy due to changes in the substrate concentration, however, may be corrected very simply if the rate of digestion is directly proportional to the concentration of substrate when the concentration of the latter is low. Experiment shows that this is actually the case. (The effect of the substrate concentration is at present under investigation.) The active concentration of substrate at any moment then will be $A-x$, where A is the original total concentration of substrate and x is the amount transformed. This has already been done in equation (5)

$$\frac{dx}{dt} = \frac{KE(A-x)}{x}$$

which on integration becomes

$$\frac{A \ln \frac{A}{A-x} - x}{ET} = K \quad (6)$$

If the foregoing hypothesis correctly expresses the mechanism of the reaction, the results calculated from Schütz's rule and equation (6) should agree with the experimental results as soon as x has reached a value ten or fifteen times as large as the quantity of active pepsin present. Before x has reached such a value, the results calculated from equation (6) or Schütz's rule, using the values of K at which they are constant, will be higher than those found by experiment. That is, the value of K in Schütz's rule or equation (6) increases for the first 10 or 20 per cent of the total digestion. As was pointed out above, this discrepancy is due to the fact that the formulas are derived on the assumption that the relative change in the pepsin concentration is inversely proportional to the change in the peptone concentration, a condition which does not hold until the peptone is present in large excess. After this point is reached both equations should correctly represent the course of the reaction until the changes in substrate become large. After this change in substrate concentration becomes significant Schütz's rule will no longer hold since there is no term in it that provides for the change in substrate concentration.[18] Equation (6) should hold (*i.e.* give a constant value for K) until the end of the reaction, since this equation takes account of the changes in substrate concentration. Table VII and Fig. 5 give the results of an experiment in which the rate of hydrolysis of an egg

[18] If the substrate concentration is high (*i.e.* more than 1 to 2 per cent) Schütz's rule will be found to hold throughout the greater part of the reaction. This is due to the fact that the rate of digestion in concentrated solutions is nearly independent of the substrate concentration. The falling off in the rate of reaction is therefore almost entirely due to the changes in the pepsin concentration. This change is correctly expressed by Schütz's rule. In high concentration of albumin Schütz's rule therefore fits better than Arrhenius' equation.

albumin solution has been followed by means of the conductivity. The total change was $\frac{1}{170}$ reciprocal ohms. The figures have been calculated to the basis of 1,000. The results show that the equations

TABLE VII.

Rate of Hydrolysis.

Substrate = 2.0 per cent egg albumin solution, pH 2.0, = 10.0 units of peptone per cc. when completely hydrolyzed (from Table III).

Pepsin = 0.02 per cent = 0.2 units per cc.

Temperature 38°C. A = 1,000.

Time. Hrs. × 10^2	Increase in resistance. = X	Values of $K = \frac{X}{\sqrt{ET}}$	Values of $K = \frac{A \ln \frac{A}{A-X} - X}{ET}$
1	1	1	0.06
7	43	16	0.14
11	62	18.7	0.23
18	118	28	0.42
22	143	30	0.51
26	183	36	0.73
31	212	38	0.85
36	240	40	0.94
41	260	40	1.00
51	282	39.5	1.00
61	335	43	1.17
73	360	42	1.17
97	415	42	1.17
125	454	40	1.18
260	582	36	1.12
362	652	34	1.10
462	690	32	1.0
562	740	31	1.1
3,600	1,000		

fit the experimental results almost exactly as was predicted from the derivation. The decline in the value of the constant near the end of the reaction of equation (6) is due to the fact that the changes in conductivity of the solution do not accurately represent the digestion at the end of the experiment.[19] It will be shown later that, when

[19] It has been shown in another paper in this *Journal* (Northrop, J. H., *J. Gen. Physiol.*, 1919-20, ii, 475) that the destruction of pepsin under the conditions of this experiment is so slight as to be negligible. The decrease in the rate of reaction cannot be ascribed to this cause.

the changes in digestion are followed by means of the increase in amino nitrogen, which probably accurately follows the digestion, equation (6) gives a constant value for K.

Equation (6) is identical with that derived by Arrhenius[16] from the action of ammonia on a great excess of ethyl acetate, and applied by him to peptic digestion. Arrhenius, however, considered A as representing the concentration of ammonia (which would correspond to the concentration of pepsin in these experiments). In other words

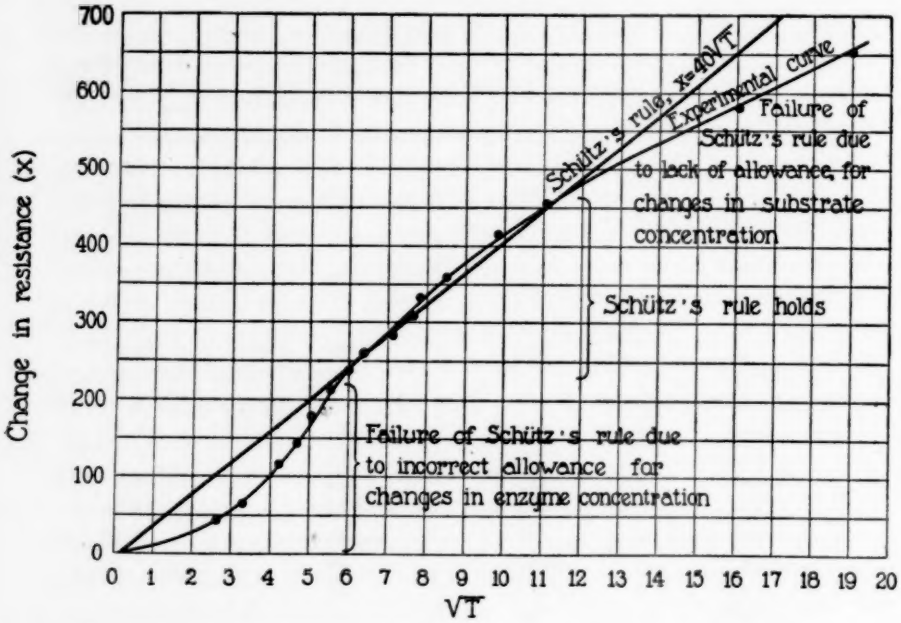
Change in resistance (x)
700
600
500
400
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
√T
Schütz's rule, x=40√T
Experimental curve
Failure of Schütz's rule due to lack of allowance for changes in substrate concentration
Schütz's rule holds
Failure of Schütz's rule due to incorrect allowance for changes in enzyme concentration

FIG. 5. Curves showing rate of digestion of egg albumin (*cf.* Table VII).

the entire term $\frac{(A-x)}{x}$ in Arrhenius' equation represents the ammonia (or enzyme) concentration while the substrate concentration is considered to remain constant. It is clear from the derivation of the equation presented in this paper, however, that the term $\frac{E}{x}$ represents the enzyme concentration, while $A-x$ represents the substrate concentration. The equation as applied to the hydrolysis of ethyl

acetate by a small amount of ammonia or to the hydrolysis of protein by pepsin is therefore identical in form but differs as to the significance of the term $A-x$.

In all the foregoing experiments the rate of digestion has been followed by means of changes in the conductivity of the solution. Since this value does not accurately represent the course of digestion, the objection might be raised that the agreement between observed and calculated values is due to compensating errors in the derivation of the equation and in the deviation of the conductivity changes from the actual progress of digestion. This could not be the case in the tests of the equation in the differential form since in this case the results are comparative and any deviation of the conductivity changes from the true rate would cancel out. It is possible, however, that the agreement of the equation in the integral form might be due to some such compensation of errors. In order to show that this is not the case a series of experiments was made in which the course of digestion was followed by means of the increase in amino nitrogen. This value was determined by Van Slyke's[20] method as already described,[21] and, as far as is known, accurately represents the progress of digestion. In these experiments the quantity of egg albumin was kept constant (0.5 per cent egg albumin) and the concentration of pepsin varied. The results are summarized in Table VIII. The figures given under X are the increase in amino nitrogen in cc. per 660 cc. of solution. They are the average of three determinations and have an experimental error of about 10 per cent. This is sufficient to account for the variations in the constant of equation (6). The errors in x are greatly magnified in this constant as it depends on the difference between two experimental values.[22] In every case sufficient time had elapsed before the first observation so that x at the time this determination was made was already large compared to the concentration of pepsin. The change in value of the constants for the first minutes of the re-

[20] Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 275.

[21] Northrop, J. H., *J. Gen. Physiol.*, 1918–19, i, 607.

[22] The errors in x are reduced in the constant of Schütz's rule. The variations in this constant are therefore outside the limits of experimental error while those of Arrhenius' constant are within the limits of experimental error.

TABLE VIII.

Comparison of Schütz's Rule and Arrhenius' Equation. Rate of Hydrolysis.

Substrate = 0.5 per cent egg albumin solution, pH 2.0.

X = relative increase in amino nitrogen per cc. of solution.

E = relative pepsin concentration.

Time.	X	E	$K = \frac{X}{\sqrt{E\,T}}$	$K = \frac{\left[A \ln \frac{A}{A-X} - X\right]}{E\,T}$
min.				
30	380	32	12.0	0.10
75	500	32	10.0	0.08
135	640	32	10.0	0.08
255	700	32	8.5	0.05
495	806	32	6.6	0.05
840	860	32	5.0	0.04
1,740	975	32	4.0	0.05
4,200	[1,000]	32		
30	224	16	10.0	0.06
75	382	16	12.0	0.07
135	528	16	11.0	0.10
255	608	16	10.0	0.07
495	620	16	7.0	0.04
840	700	16	6.0	0.04
1,740	860	16	5.0	0.04
4,200	[1,000]	16		
30	152	8	10.0	0.06
75	277	8	11.0	0.08
135	390	8	12.0	0.10
255	516	8	11.0	0.10
495	536	8	12.0	0.06
840	656	8	8.0	0.05
1,740	742	8	6.5	0.06
4,200	930	8	2.3	0.07
30	100	4	13.0	0.045
75	174	4	10.0	0.055
135	225	4	11.0	0.055
255	363	4	11.0	0.08
495	415	4	9.4	0.06
840	490	4	8.5	0.055
1,740	636	4	7.6	0.055
4,200	770	4	6.0	0.045

TABLE VIII—*Concluded.*

Time.	X	E	$K = \frac{X}{\sqrt{ET}}$	$K = \frac{\left[A \ln \frac{A}{A-X} - X\right]}{ET}$
min.				
30	90	2	11.5	0.07
75	139	2	11.5	0.07
135	180	2	11.0	0.07
255	265	2	12.0	0.06
495	305	2	10.0	0.06
840	385	2	9.5	0.06
1,740	500	2	8.5	0.06
4,200	630	2	7.0	0.05
30	80	1	14.5	0.10
75	93	1	11.0	0.06
135	112	1	10.0	0.05
255	165	1	10.0	0.06
495	180	1	8.0	0.03
840	230	1	8.0	0.04
1,740	350	1	8.5	0.05
4,200	420	1	6.5	0.03

action, noticed in Table VII, is therefore lacking here. The results show that the equation gives a fairly satisfactory constant when it is considered that the experimental observations are very difficult and that the experiments represent changes in the value of E, x, and T of many hundred per cent. Individual experiments were made which gave much more constant values for K. The present series is given preference, however, since it shows that the equation takes into consideration changes in the enzyme concentration. It is obvious, however, that this equation is merely an approximation formula which will hold only under certain limited conditions and is but little more general than Schütz's rule. The derivation given offers a rational interpretation of both expressions. It may be pointed out also that equation (6) contains only one arbitrary constant K and can therefore hardly be considered as empirical.

DISCUSSION.

It has been shown in the preceding paragraphs that the divergence of the kinetics of pepsin action, from the results predicted from the

law of mass action, may be quantitatively explained by the assumption that the enzyme in solution is in equilibrium with the products of digestion of the protein, or some other substance, and that this equilibrium obeys the ordinary laws of mass action. The results of Peckelharing and Ringer[23] may be taken as experimental proof that the enzyme is so combined. These authors found that very pure solutions of pepsin showed no isoelectric point when tested between two oppositely charged electrodes; but that the addition of peptone caused the pepsin to change the direction of migration at a pH of about 3.0; which corresponds approximately to the isoelectric point of these added substances. It is difficult to explain this experiment otherwise than to conclude that the pepsin combines with the peptone and is carried with it to the electrode. If some of the pepsin combines with peptone, therefore, and so becomes inactive the rate of digestion will evidently not be directly proportional to the total concentration of pepsin but to some other function of the total concentration as defined by the mass action equilibrium. This is exactly analogous to the relation between the hydrogen ion concentration and the total acid concentration. In this case it is only the hydrogen ion which is active in hydrolysis and the activity of the solution is therefore not directly proportional to the total acid concentration. (In the case of acid it is known that the dissociation is electrolytic; *i.e.*, the dissociated parts of the molecule are electrically charged. Whether this is true or not in the case of the pepsin cannot be stated as yet.) The rate of reaction then becomes directly proportional to the active (free) enzyme concentration as demanded by the law of mass action; and the apparent divergence from this law is due to the fact that the total enzyme concentration and the active enzyme concentration are not always directly proportional; just as the total acid concentration and the active acid concentration are not always directly proportional. If this hypothesis is correct, it seems probable that the enzyme does not combine with the substrate for an appreciable length of time, but that the contact of enzyme and substrate molecule results in immediate decomposition of the latter into its products of digestion. There is no doubt that the enzyme actually does combine with the substrate when the latter is not in solution.[6] It is quite possible, however, that this is a case of solution of the en-

[23] Peckelharing, C. A., and Ringer, W. E., *Z. physiol. Chem.*, 1911, lxxv, 282.

zyme in the solid phase and that the kinetics of the reaction are the same there as in the liquid phase. There is some experimental evidence in favor of this point of view. It was found that the rate of digestion of edestin was the same when in solution and when suspended in the enzyme solution.[21] Dauwe[24] has shown that pepsin can diffuse through a membrane of solid protein.

The hypothesis enables us to set an upper limit for the purity of an enzyme preparation. It was found, for instance, in Experiment 1, that the enzyme solution used contained about twenty-seven (arbitrary) units of pepsin and about thirty units of peptone. Assuming that the combining weights of the substances are approximately the same it is obvious that the original preparation could not have been more than 50 per cent pure pepsin. It is, however, quite possible that the enzyme may be combined with some substance and still retain its activity (as found for invertase combined with charcoal by Nelson and Griffin[25]) or that impurities are present which do not combine with the enzyme at all. It is not possible therefore to assume that the active pepsin consists solely of pepsin molecules. For similar reasons it is not possible to draw any definite conclusions from the results of Experiment 2 in which it was found that a 1 per cent egg albumin solution after complete digestion contained about two arbitrary units of peptone while a 1 per cent pepsin solution contained about ten units of pepsin.

It is well known that the kinetics of enzyme reactions differ in another respect from the general laws of chemical reactions in that the rate of reaction in high concentration of substrate does not vary directly with the total substrate concentration. This phenomenon is very similar to the one discussed in the present paper and it would seem that the same explanation applies to both cases; *i.e.*, that the active substrate concentration is not directly proportional to the total substrate concentration.

It may be pointed out that, according to the above mechanism of the reaction, pepsin cannot be considered a catalyst in the sense of the classical definition since it combines with some, at least, of the products of reaction and so enters directly into the equation. Since

[24] Dauwe, F., *Beitr. Chem. Physiol. u. Path.*, 1905, vi, 426.

[25] Nelson, J. M., and Griffin, E. G., *J. Am. Chem. Soc.*, 1916, xxxviii, 1109.

the enzyme combines with one (at least) of the products of reaction its presence must necessarily affect the equilibrium point. The reaction, therefore, would appear to be a special case of bimolecular reaction in which one of the reacting substances (the enzyme) forms a highly dissociated compound with one of the products. The truth of the matter probably is that so called pure catalytic reactions are merely limiting cases in which the combination of the catalyst is so small as to escape detection (Stieglitz).[5]

SUMMARY.

1. In certain cases the rate of digestion of proteins by pepsin is not proportional to the total concentration of pepsin.

2. It is suggested that this is due to the fact that the enzyme in solution is in equilibrium with another substance (called peptone for convenience) and that the equilibrium is quantitatively expressed by the law of mass action, according to the following equation.

$$\frac{\text{Concentration pepsin} \times \text{concentration peptone}}{\text{Concentration pepsin-peptone}} = K$$

It is assumed that only the uncombined pepsin affects the hydrolysis of the protein.

3. The hypothesis has been put in the form of a differential equation and found to agree quantitatively with the experimental results when the concentration of pepsin, peptone, or both is varied.

4. Pepsin inactivated with alkali enters the equilibrium to the same extent as active pepsin.

5. Under certain conditions (concentration of peptone large with respect to pepsin, and concentration of substrate relatively constant) the relative change in the amount of active pepsin is inversely proportional to the concentration of peptone and the equation simplifies to Schütz's rule.

6. An integral equation is obtained which holds for the entire course of the digestion (except for the first few minutes) with varying enzyme concentration. This equation is identical in form with the one derived by Arrhenius[16] for the action of ammonia on ethyl acetate.

7. It is pointed out that there are many analogies between the action of pepsin on albumin solutions and the action of toxins on an organism.

THE DARK ADAPTATION OF THE HUMAN EYE.

By SELIG HECHT.

(*From the Physiological Laboratory, College of Medicine, Creighton University, Omaha.*)

(Received for publication, February 10, 1920.)

I.

The phenomenon of retinal adaptation is one of the most familiar facts of sensory physiology. Not only is the mere adaptability of the human eye well known, but, since Aubert's (1865) first measurements, there has grown up a body of quantitative data describing the *course* of adaptation (Nagel, 1911). This is especially true of the adaptation of the eye to dim lights.

All the data on dark adaptation show that on entering a dark room after a stay in the outside daylight the eye at once begins to increase in sensitivity. At first this increase appears to be slow; but after 5 minutes the increase is quite rapid, the eye acquiring a sensitivity several hundred times its initial value. After 30 minutes sojourn in the dark the sensitivity still increases, but more slowly than before; and after 45 minutes or an hour the maximum sensitivity is reached. The final sensitivity varies slightly with different people, but in the fully adapted condition the eye is easily 5,000 or 10,000 times more sensitive than it was at the beginning.

In Fig. 1 is given the record of a dark adaptation experiment made by Piper (1903). The results are representative of the numerous published experiments. Piper points out that, although earlier workers (*e.g.* Aubert, 1865) believed the rate of adaptation to be greatest at first, the greatest increase in sensitivity really occurs in the middle of the course of adaptation. The curve of sensitivity according to Piper shows three parts: an initial slow phase, an intermediate rapid one, and a final phase ending in a maximum.

In spite of our familiarity with the phenomena of dark adaptation, and the trustworthiness of the measurements describing them, their

theoretical bearing is practically *nil*. The changes in sensitivity are remarkably constant, not only in a given individual, but in different individuals as well. Age does not change the regularity of the course. The effect of drugs is practically negligible. Even persons possessing deficiencies of color vision present a normal type of dark adaptation. Still the meaning of this uniformly regular change in sensitivity has remained obscure. The course of dark adaptation has not given us a hint of the physicochemical basis of visual reception, though it is apparent that the two phenomena must be fundamentally related to each other.

What are the causes of this failure? In order to answer this question profitably we must consider first the nature of the published data, and second the obstacles in the way of their interpretation inherent in the data. Extensive summaries of the literature of retinal adaptation have been made (Tschermak, 1902; Nagel, 1911). Such is not my purpose. It is rather to analyze the data, and if possible to find some explanation of the pronounced regularity evident in every experiment on the dark adaptation of the human eye.

II.

The pioneer experiments of Aubert (1865), followed by those of Charpentier (1886) and Treitel (1887), demonstrate the qualitative fact that dark adaptation follows a definite course. Due to matters of technique, however, they are not sufficiently accurate to stand on a par with the later experiments of Piper (1903) and Nagel (1911). We shall therefore confine ourselves to the work of the latter investigators. Piper in particular has published the complete results of the retinal adaptation of eighteen people. These detailed data are invaluable in the quantitative treatment of the material.

The experiments consist in finding the intensity of a square area of light which is just barely visible to the eye. Observations are made at regular intervals during the stay in the dark. The subject fixes his eyes on one corner of the square of light, so that most of the light falls on the retina outside the fovea centralis. What one finds is this. At first this minimum intensity is large; as the stay in the dark is prolonged it becomes less and less; and finally it reaches a constant minimum.

Not content with the mere statement of such facts, the investigators beginning with Piper have presented their data in terms of sensitivity or *Empfindlichkeit*. As used in this connection these two words signify some multiple of the reciprocal of the minimum intensity. The actual units of sensitivity vary. Piper uses a million times the reciprocal, whereas Nagel considers *Empfindlichkeit* as the simple reciprocal of the minimum intensity. It is apparent, however, that the two are essentially the same thing. The data shown in Fig. 1 are given in terms of Piper's units of sensitivity.

It is here that we meet the first difficulty. Sensitivity as defined in this way possesses no meaning other than that inherent in the original fact of the minimum intensity. It is true, speaking in a general way, that the irritability of the eye increases as the minimum intensity necessary to stimulate it decreases. But we must not be deceived by so seducive a word as sensitivity, even when it is accompanied by certain figures purporting to represent the number of units of this condition. It is so easy to forget this, and to apply the term as a quantitative estimate of the condition *inside* the eye (*Empfindlichkeit der Netzhaut*) instead of remembering that it applies merely to the condition of the *outside* light. *Empfindlichkeit* includes nothing more than what is implied in the minimum intensity necessary to elicit a visual effect in the eye.

However, even as a statement of the changes in the external light, the use of *Empfindlichkeit* or sensitivity is attended with the danger that it distorts the actual course of retinal adaptation. As a matter of fact the shape of the curve in Fig. 1 and its division into three phases represent neither the properties of the retina nor those of the light. It does represent a certain property of numbers. The whole thing depends on the simple fact that, as a number decreases, its reciprocal increases in a curious way. For example

$$y=x$$

is the equation of a straight line. However

$$y=\frac{1}{x}$$

that is, using the reciprocal of x is not the equation of a straight line at all, but that of an equilateral hyperbola.

From Fig. 1 it might really be supposed that practically no change occurs during the first 5 minutes of dark adaptation. Nothing is farther from actual fact. During this interval the minimum intensity necessary for a visual stimulus,—the visual threshold,—drops to less than half its initial value. In the experiment of Fig. 1 this

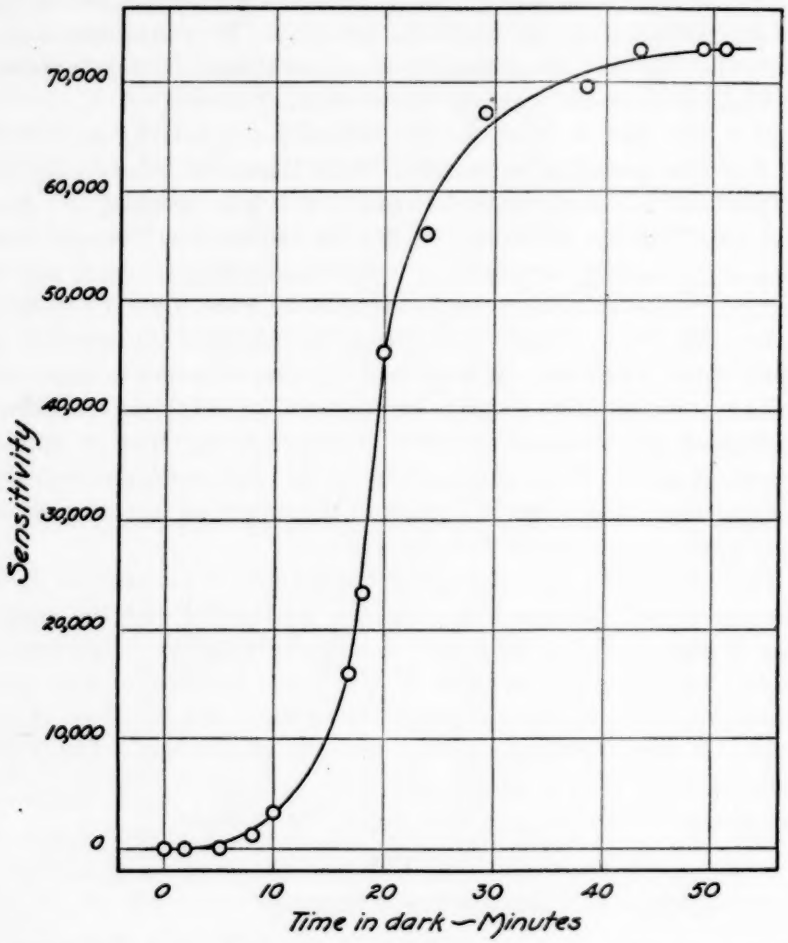

FIG. 1. The course of dark adaptation expressed in terms of the increase in sensitivity. This experiment is typical of the many which have been published. The ordinates are a million times the reciprocal of the minimum intensity; the abscissæ the time in the dark.

decrease amounts to no less than 61 per cent of the initial threshold value. A glance at Fig. 2 and at Table I, presented later in this paper, shows that the minimum intensity,—the data actually obtained,—varies in no such way as indicated by Fig. 1. The data of Fig. 2 are the same as those forming the basis of Fig. 1.

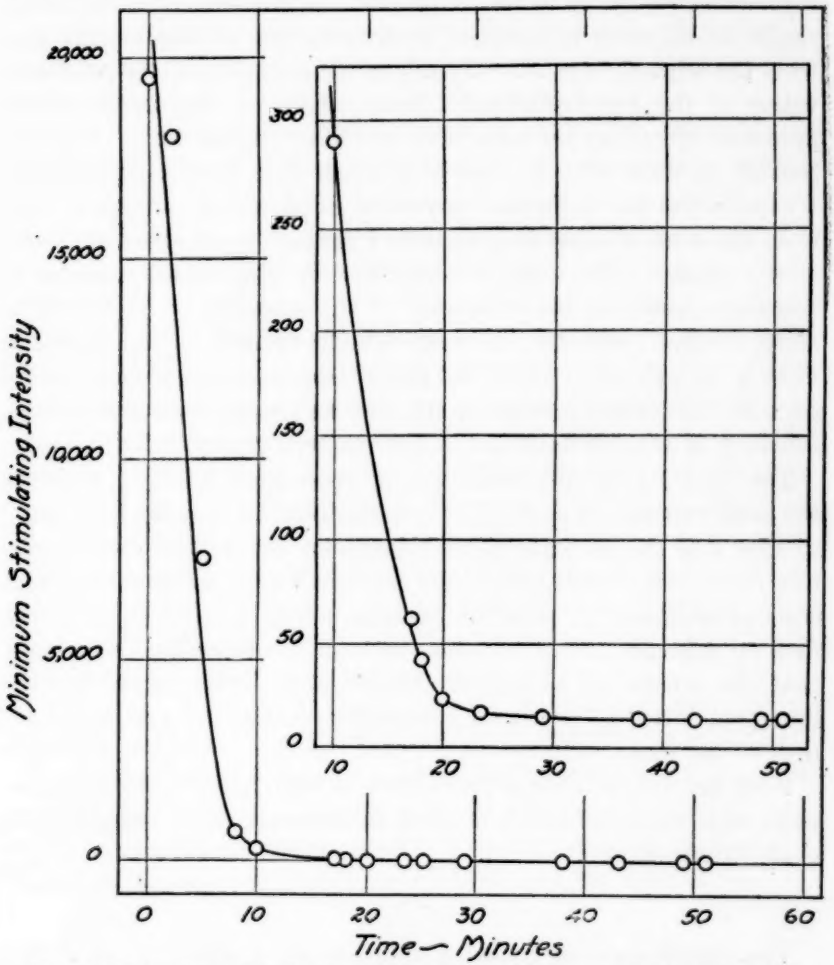

FIG. 2. The course of dark adaptation expressed in terms of the minimum intensity visible to the eye. The data are the same as in Fig. 1. The lower portion of the curve is redrawn in the inset, using a magnified scale of ordinates. The exceedingly great change in the intensity during the first 10 minutes is clearly apparent.

Unfortunately all the investigators following and including Piper have published their results as *Empfindlichkeit*, without giving the minimum intensities as they found them. This has been the most obvious impediment in the way of a proper treatment of their data. The undue emphasis on the distorted results has detracted from the regularities evidenced by the actual data themselves. It is, however, simple in all cases to compute backwards and to find in this way what the original facts are. By taking the reciprocal of the published values of the *Empfindlichkeit* I have calculated the corresponding minimum intensities for most of the published experiments. It is not possible to state what the unit of intensity is in Piper's experiments. I cannot find its definition anywhere in his article. By inference from the work of other investigators I judge it to be about 4×10^{-7} meter candles. The unit of sensitivity in Nagel's adaptometer is definitely stated as the reciprocal of the intensity as measured in meter candles. However, in order to make these data comparable to Piper's, as well as to avoid the use of long decimals, I have multiplied the minimum intensity by 10^7, thus making the unit of minimum intensity in Nagel's data as 1×10^{-7} meter candles.

The results as we find them now are represented by Fig. 2, which is the same experiment as Fig. 1. It is not possible in a single drawing to show how the intensity varies throughout the test. I have therefore redrawn the lower part of the curve in Fig. 2 using a magnified scale of ordinates to show the changes which take place after the first 10 minutes. It is obvious, as Aubert originally maintained, that the process of adaptation begins immediately, and that the minimum intensity decreases enormously during the first few minutes in the dark.

With the data in their present form we may now proceed to determine what the peculiarities inherent in them are which prevent their ready interpretation.

III.

The experiments on retinal adaptation are a series of determinations of the visual threshold in dim light. Each test is a measurement of the minimum energy for a sensory effect. The interpretation of the findings must then hinge to a large extent on the phenomena that

attend the sensory reception of lights of low intensity. At present we know very little of the basic reactions, chemical and physical, that underlie retinal stimulation. One thing, however, may be stated with considerable confidence. This is that the initial effect of the light on the retina consists of the photochemical alteration of some photosensitive substance. Each test of the visual threshold involves a primary photochemical effect. The data of dark adaptation, if properly treated, should give us information about this photochemical change.

Objectively stated, the facts are that as adaptation proceeds, less and less light energy is necessary to produce the initial photochemical effect required for a visual response. In other words, progressively less and less of the photosensitive substance must be decomposed in order to initiate the subsequent processes concerned in photoreception.

Granting this qualitative decrease, what are its quantitative implications? We have the minimum intensities from the data. What is the relation between the intensity of the stimulating light and its objective effect in the photochemical decomposition of the sensitive material of the retina? An exact relation undoubtedly exists between the two. Its quantitative expression must be known if the adaptive changes in the retinal mechanism are to be stated objectively. Unfortunately this condition cannot be fulfilled from any experiments on the retina. We have here, then, the first of the inherent difficulties in our knowledge that prevent the proper fundamental interpretation of retinal adaptation.

This, however, is not the only obstacle. Let us tentatively assume that the relation between intensity and photochemical effect is known quantitatively. In this way we will know the exact quantities of photosensitive substance required to be decomposed in order to initiate a minimal retinal effect. This follows from the fact that the photochemical effect E is some function of the intensity I

$$E=f(I)$$

even though the exact nature of the function is not stated.

During adaptation in darkness the amount to be decomposed changes regularly. What determines the magnitude of the quantity

of decomposed material necessary for the initiation of a visual effect at a given moment? This really amounts to a demand for the objective basis of variations in the irritability of the retinal mechanism. We must know why at any given moment a certain number of units of decomposed photosensitive material is necessary for the production of a visual response, before the strikingly regular variations of these quantities can attain any basic significance. Here we meet with the second obstacle in the way of an interpretation, because the question cannot be cleared up in terms of the existing data of retinal physiology. Indeed it is difficult to conceive of experiments on the vertebrate retina so designed as to give an objective answer to these two questions.

It must be remembered that the important point of the data of retinal adaptation is not merely the fact of adaptation, but the consistently regular sequence in the course of adaptation. Given the means of answering the two questions relating to visual reception, this orderly progress of dark adaptation might be attacked with profit. Lacking them, it is small wonder that the data are meaningless in themselves, and that they have failed to add to a possible hypothesis for the basis of visual reception.

IV.

Although retinal physiology has not been able to surmount the difficulties previously enumerated, there are some experiments recently made with invertebrates that may help in this connection. The work on the light sensibility of *Mya* and *Ciona* (Hecht, *a*, *b*, *c*, *d*) has demonstrated two aspects of the sensory process which are intimately connected with the problem of retinal adaptation. The first of these is concerned with the relation between the intensity of the stimulating light and its photochemical effect in photoreception. The second presents an objective basis for the meaning of variations in irritability.

According to the hypothesis suggested for it, the photosensory mechanism in *Mya* is composed of two processes, one following the other in point of time. The initial process is a photochemical reaction; the subsequent one is an ordinary chemical reaction which is

catalyzed by the products formed in the photochemical reaction. It is the initial photochemical reaction that is of interest in the present context, and with it alone we shall be concerned. Without going into details,—for which the original work must be consulted,—the results are as follows. A photosensitive substance S is decomposed by the action of light into two products of decomposition P and A. This reaction is reversible, and in the absence of light the reverse reaction goes on unopposed by the light reaction. The equation for the complete process is thus

$$S \underset{\text{"dark"}}{\overset{\text{light}}{\rightleftharpoons}} P + A.$$

It is apparent that the substances P and A are the precursors as well as the decomposition products of the photosensitive substance S.

The velocity of the light reaction, $S \rightarrow P + A$, is entirely dependent on the intensity of the incident light. The exact relation between the two is a logarithmic one. If E is the photochemical effect, as measured by the amount of P and A formed in unit time, and I the intensity of the light, then

$$E = k \cdot ln\, I$$

ln being the sign of natural logarithms. In *Mya*, k has a value of 1. This quantitative relation is the first of the two aspects of the sensory process to which reference has been made. Its significance lies in its ability to describe the action of light in the objective terms of a physicochemical mechanism, rather than in terms of sensory effects.

The second feature of the photosensory mechanism as postulated for *Mya* and *Ciona* is also concerned with products of the light reaction $S \rightarrow P + A$. This second principle states that the degree of irritability of the sense organ depends entirely on the concentration of the precursor decomposition products present in the sensory mechanism. To be more precise: before it can cause a sensory effect, the incident light must produce such an amount of freshly decomposed precursors P and A that a definite ratio is attained between the freshly formed and the residual precursors present in the sense organ.

The importance of this concept cannot be overemphasized, because it lies at the foundation of all the work with *Mya* and *Ciona*. Together with the logarithmic relation previously explained, it enables one to visualize the initial chemical events necessary for the production of a sensory effect in these animals.

V.

If the initial photochemical reaction in retinal sensitivity has something in common with photoreception in *Mya*, then the application of the findings with *Mya* to the data of visual adaptation should yield results of theoretical bearing. The data give the intensities of the light necessary for a visual effect. The photochemical action of this light should therefore be represented by the logarithm of its intensity. These logarithmic values will then give the actual number of units of photosensitive substance decomposed by the light, because

$$E = k . \log I.$$

We do not know what the value of the constant k is here. But we can always make it equal to 1 by changing the units in which the photochemical effect is measured. Such a change makes no difference in our conclusions, because any unit is a purely arbitrary thing.

The data treated in this way are given in Figs. 3 to 8. These figures are different experiments taken from the work of Piper (Figs. 3 to 7) and of Nagel (Fig. 8). An example of the procedure necessary in calculating the data from the published experiments is given in Table I. The first two columns are from the published results. The last two columns are the computed values of the original intensities and their logarithms. The data of Table I are given graphically in Fig. 3.

In the presentation of these experiments I have exercised a certain amount of selection in the following way. Some of Piper's experiments are vitiated by the fact that the measurements are made too frequently. For example, in one case nine determinations are recorded in 11 minutes, some having been made only ½ minute apart. Considering that even a small flash of light retards dark adaptation, it is hard to understand why such an error should have been com-

TABLE I.

Data of an Experiment on Dark Adaptation of the Eye.

Time.	Sensitivity.	Intensity.	Logarithm of intensity.
min.			
0	38.7	25,840.0	4.41
2	99.7	10,030.0	4.00
5	398.7	2,506.0	3.40
8.5	1,065.0	934.6	2.97
12	3,420.0	292.4	2.47
20	16,870.0	59.9	1.78
31	30,780.0	32.5	1.51
39.5	45,270.0	22.1	1.34
45	54,080.0	18.5	1.27
72	54,080.0	18.5	1.27

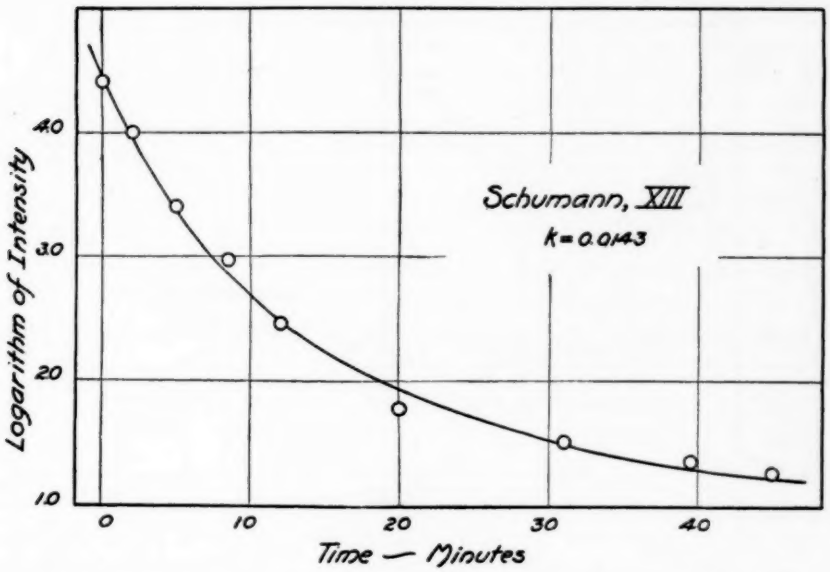

FIG. 3. Dark adaptation of the eyes of Dr. Schumann, as determined by Piper. Schumann has an anomalous trichromatic vision.

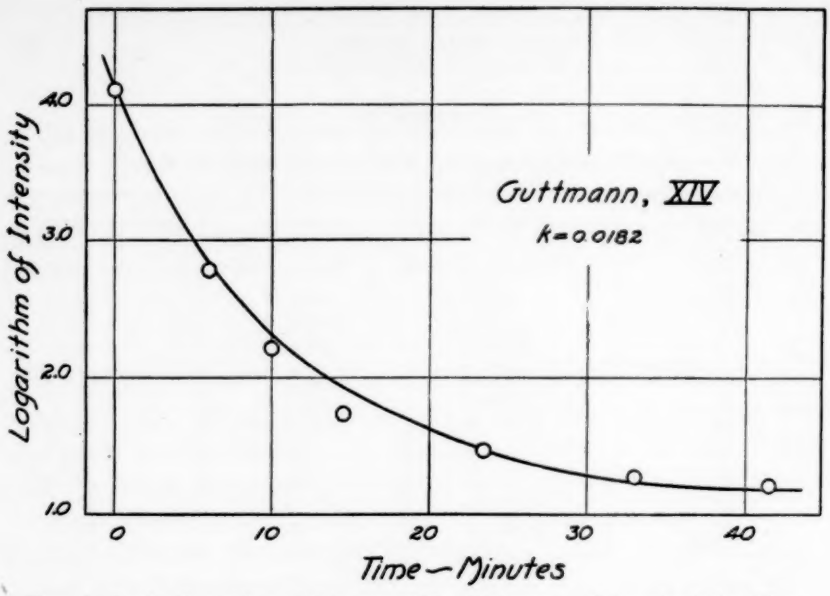

FIG. 4. Dark adaptation of the eyes of Dr. Guttmann, determined by Piper. Guttman is also an anomalous Trichromat.

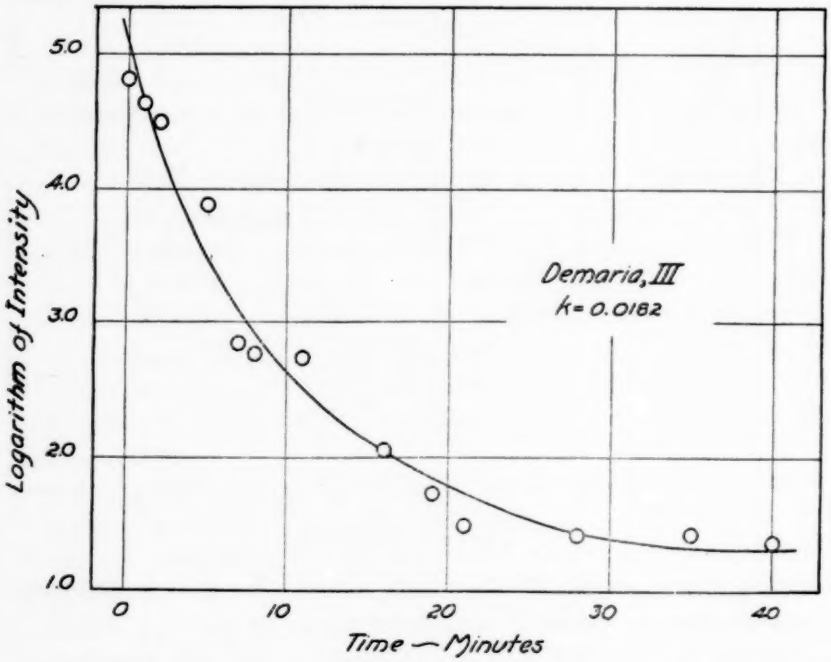

FIG. 5. Dark adaptation experiment with Dr. Demaria as subject. Normal trichromatic vision. Piper's data.

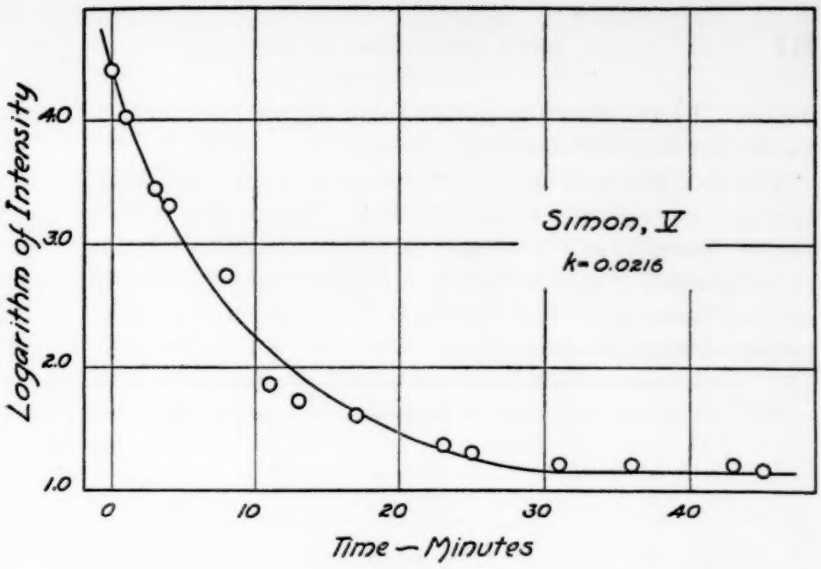

FIG. 6. Dark adaptation of Dr. Simon's eyes, determined by Piper. Simon's vision is that of a normal Trichromat.

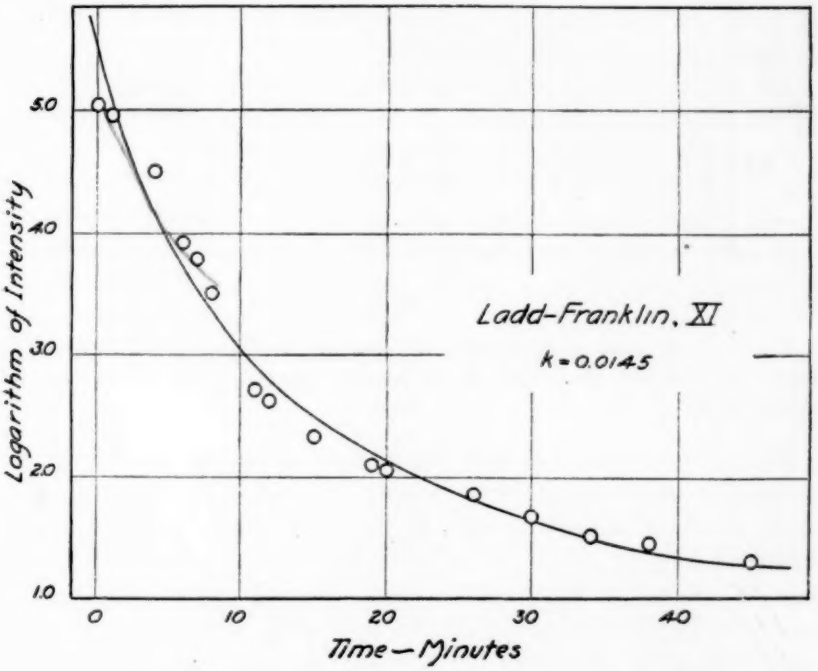

FIG. 7. Adaptation experiment with Mrs. Ladd-Franklin as subject. Normal trichromatic vision. Piper's data.

mitted. I have therefore chosen those experiments which are practically free from this error of technique.

The ordinates in Figs. 3 to 8 are given as the logarithms of the intensity in order to present the data. They really must be interpreted, however, as the number of units of photochemical decomposition products freshly formed by the light, because of the logarithmic relation between the two. As such, what do they tell us about the process of adaptation?

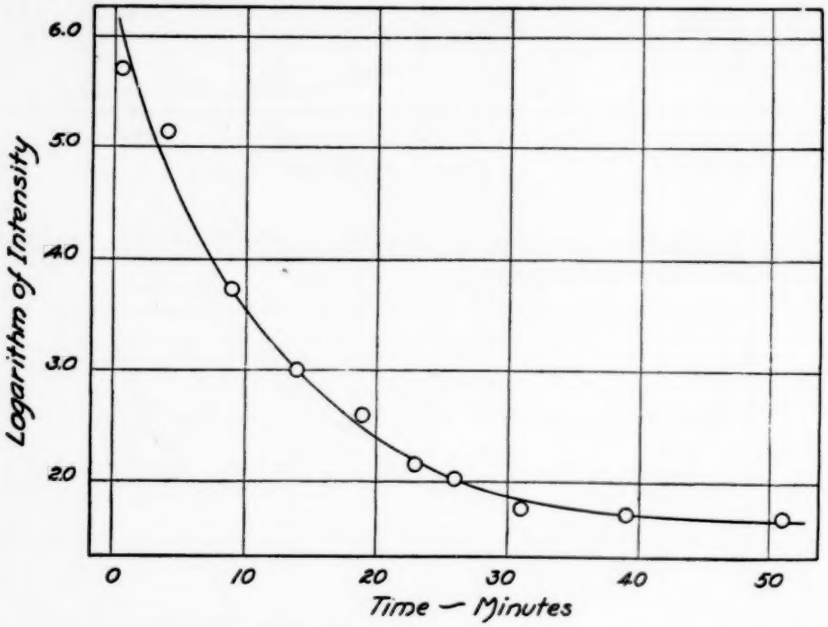

FIG. 8. Dark adaptation experiment recorded by Nagel. The units of intensity are different from that of the previous figures. The results, however are the same.

We learned from *Mya* that the amount of freshly formed decomposition products must be a definite proportion of the amount of these products already present in the sense organ before they can initiate a sensory effect. If we know the required amount of freshly formed products, we therefore know the amount of the residual products, because the ratio between the two is constant. The necessary quantities of freshly decomposed photosensitive material are

thus a direct measure of the amount of decomposition products at that moment in the retina. The points in Figs. 3 to 8 then give the concentration of the residual decomposition products.

The data now assume a dynamic aspect. Each figure represents the changes in the concentration of the residual decomposition products during adaptation. It is apparent that the concentration steadily decreases. The decrease is regular and follows the course of a chemical reaction. In order to show this the curves in all the figures are the isotherms of a bimolecular reaction. The equation for the curves is

$$k=\frac{1}{a\cdot t}\cdot\frac{x}{a-x}$$

the values of k for each experiment being given in the respective figures. It must be emphasized that each of the points in these figures represents only a *single* measurement. The agreement between the individual experimental values and the theoretically calculated curves is truly striking.

The fact that the curves are reactions of the second order is significant. *Two* products of decomposition are diminishing in concentration in a manner which shows that they are combining to form a chemical compound. These two products are originally the results of the decomposition of a photosensitive substance. A simple explanation is that the compound formed by their chemical union is identical with the photosensitive substance from which they were formed. Such an interrelation is quite common in photochemical reactions. Using S to denote the photosensitive substance, P the principal product of decomposition, and A its accessory, we may write

$$S \underset{\text{"dark"}}{\overset{\text{light}}{\rightleftarrows}} P + A$$

as the equation of the photochemical reaction. This reaction forms the initial step in the visual reception of dim light by the human retina. The substances P and A are thus the precursors as well as the decomposition products of the photosensitive substance S, the three forming the components of a completely reversible reaction (*cf.* Hecht, 1918–19, *a*, p. 161).

VI.

In terms of such a reversible photochemical process the phenomena of dark adaptation are fundamentally simple. During the stay in the light, a large amount of photosensitive substance is decomposed according to the reaction $S \rightarrow P + A$. The rate of this decomposition will depend entirely on the intensity of the light. The reverse, "dark" reaction $P + A \rightarrow S$, being an ordinary chemical reaction, will proceed whenever any products of decomposition are formed. According to the mass law the rate of synthesis of S from P and A will depend on the concentration of the latter two substances. Between the two opposing reactions a stationary state will be reached (*cf.* Weigert, 1911, p. 15, for the difference between a stationary state and a condition of true equilibrium). This stationary state will represent a definite concentration of the three components, and will depend entirely on the light intensity.

Removal into the dark at once causes the light reaction, $S \rightarrow P + A$, to stop, leaving the "dark" reaction, $P + A \rightarrow S$, to go on unopposed. The continuous action of this "dark" reaction then determines the course of dark adaptation. In order to measure the irritability during dark adaptation a visual test is used. The necessary light decomposes S into fresh P and A. The ratio between fresh and residual P and A is constant. Therefore as the residual products of decomposition disappear, less and less fresh P and A are required to initiate a visual effect. In other words, the retina becomes more and more irritable, as we already know.

The fact that the eye may become adapted to any intensity of light finds its explanation in the stationary state of the opposing chemical reactions. This is entirely a function of the intensity of the light, provided the temperature remains constant. At high intensities the concentration of P and A during the stationary state will be much greater than at lower intensities. This means that the light required to produce a minimum visual effect will vary similarly. Thus the sensory threshold will be higher at higher intensities than at lower, which again is a truism of retinal physiology (*cf.* Hecht, 1918–19, *b*, p. 553).

It is not my purpose to show how many of the properties of visual reception may be explained in terms of the reversible photochemical reaction postulated for the initial effect in photosensory reception. Outside of dark adaptation, this is possible only in a qualitative way, because much of the needed data does not exist in quantitative form. It is clear, however, that the consequences of the reversible reaction may be calculated and predicted, and experiments devised to test the possibilities. Such quantitative results will be forthcoming in the future, and their analysis will be reserved for that time.

One caution must be mentioned. The provisions of the Duplicity Theory make a clear distinction between vision in dim light and vision in bright light (Nagel, 1911). Dark adaptation is essentially a phenomenon of dim vision. Therefore all our conclusions must be limited to the mechanism of vision at low intensities only. The properties of photoreception at high intensities, involving as it does color vision, cannot be considered at the present time. It will be remembered that defects in color vision cannot be correlated with any changes in the course or the quantity of dark adaptation (Piper, 1903, p. 191). Indeed the results given graphically in the present paper represent not only normal Trichromats, but anomalous Trichromats as well.

The fact that our analysis applies only to vision in dim light is of considerable advantage in one respect. This concerns the final meaning of the terms in the equation

$$S \underset{\text{"dark"}}{\overset{\text{light}}{\rightleftarrows}} P + A$$

for the photochemical reaction of photoreception. With vision in dim light there has been associated the existence of visual purple. The evidence for the connection of visual purple with vision is quite striking (Trendelenburg, 1911; Henri and des Bancels, 1911). The most significant facts in this respect are those concerned with the threshold of sensitivity, the photochemical action of light on visual purple, and the absorption of energy by visual purple. It is known that spectral light falling on the retina, when so diminished in intensity as to be barely visible to the dark-adapted eye, produces a

sensation of light without color. The minimum stimulating energy at different wave lengths has been determined. Similarly the minimum energy necessary to produce a bleaching effect on visual purple has been measured. And finally the light absorbed by the pigment at different wave lengths has also been described. When put into graphic form, the curves of these three measurements all follow the same course (Henri and des Bancels, 1911). This is a powerful argument for the participation of visual purple in the process of photosensory reception.

The reversible character of the chemical behavior of visual purple is well known (Kühne, 1879). The bleached pigment in the retina, and even *in vitro* under certain conditions, regenerates its color when placed in the dark. It may therefore be quite possible that the photosensitive substance *S* which our analysis requires is really visual purple. If this is true, it follows that visual purple when bleached by light breaks down into two substances. An investigation of the dynamics of the regeneration of visual purple will furnish deciding evidence for the identification of visual purple with the hypothetical substance *S*. It is not necessary to suppose that the decomposition and the synthesis represent elaborate changes. A process of reduction, or oxidation, or perhaps of hydrolysis may accomplish all that is required in the way of chemical changes. In fact, considering the extremely small quantities of energy necessary to produce a visual effect, it must follow that the initial photochemical transformation is not only delicate but very simple as well.

The significant point of all this is that the analysis of dark adaptation with which this paper has been concerned is consistent with what we know of the changes in the eye. If further experiments will show the identity of visual purple with the hypothetical photosensitive substance, it will be a distinct advance in our knowledge of the basis of visual reception.

SUMMARY.

During the dark adaptation of the human eye, its visual threshold decreases to a small fraction of its original value in the light. An analysis of the quantitative data describing this adaptation shows that it follows the course of a bimolecular chemical reaction. On the

basis of these findings it is suggested that visual reception in dim light is conditioned by a reversible photochemical reaction involving a photosensitive substance and its two products of decomposition. Accordingly, dark adaptation depends on the course of the "dark" reaction during which the two products of decomposition reunite to synthesize the original photosensitive substance.

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THE SIGNIFICANCE OF THE CAMBIUM IN THE STUDY OF CERTAIN PHYSIOLOGICAL PROBLEMS.

By I. W. BAILEY.

(From the Bussey Institution for Research in Applied Biology, Forest Hills, Mass.)

PLATE 1.

(Received for publication, January 20, 1920.)

INTRODUCTION.

During the last few years, I have had occasion to devote considerable attention to the study of the cambium of the higher plants, in endeavoring to determine to what extent variations in the structure of timber affect certain of its physical and mechanical properties. In conducting these investigations, I have been impressed by the fact that the cambium is an unusually favorable medium for the study of certain theoretical problems, particularly the working sphere of the nucleus, the much discussed nucleocytoplasmic-relation, and the dynamics of karyokinesis and cytokinesis. It seems advisable, accordingly, to call attention to certain phenomena[1] in the cytology of this meristematic tissue which may be of general interest to physiologists as well as to cytologists.

Description of the Material.

As is well known, the stems and roots of gymnosperms and dicotyledons increase in diameter through the activity of a jacketing layer of undifferentiated tissue which forms xylem internally and phloem externally. In the case of most gymnosperms and arborescent and fruticose dicotyledons, the initials of this lateral meristem or cambium continue to divide throughout the life of the plant, and their more or less highly differentiated derivative cells constitute the bulk

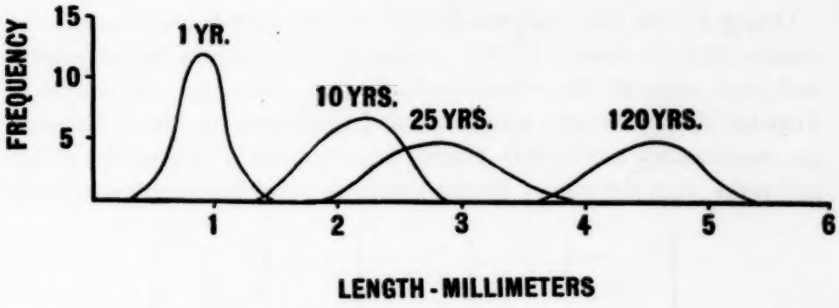
[1] These phenomena will be described and discussed in detail in a series of papers to be published in the *American Journal of Botany*.

of the tissue of an adult individual. The cambial initials are of two distinct shapes and sizes: (1) numerous, large, much elongated (parallel to the long axis of the stem or root) elements; and (2) scattered aggregations of small, more or less isodiametric cells which divide to form the horizontal sheets of radially disposed parenchyma, the so called medullary rays. The principal divisions in both types of initials are periclinal or parallel to tangents to the circumference of the stem or root. In other words, the large cells divide in a tangential longitudinal plane, which is a division plane of *maximal* area, whereas the ray initials form partitions that commonly are surfaces of *minimal* area.

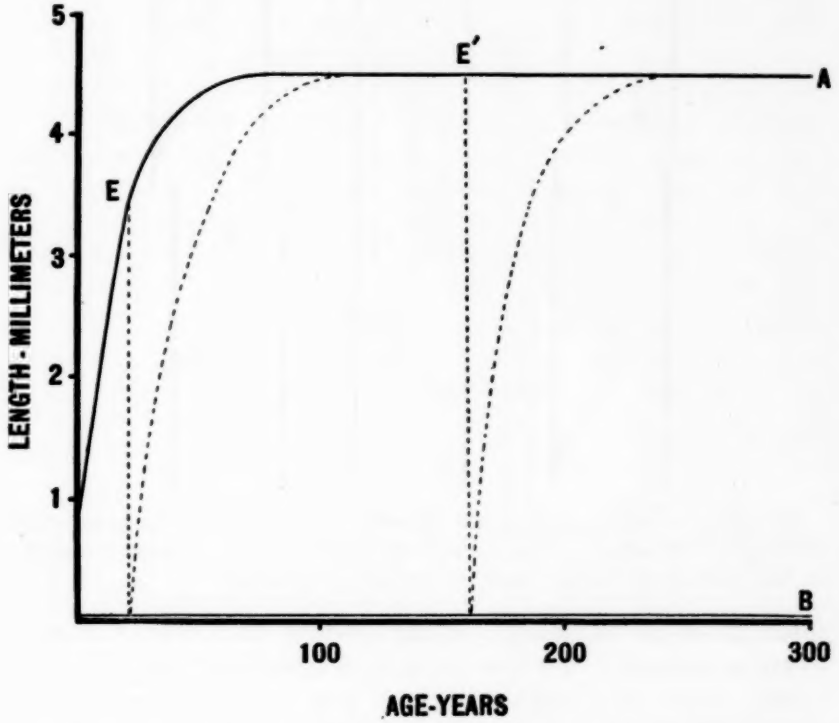
The tangential diameter of the cambial initials increases to a certain extent during the earlier stages of the enlargement of stems and roots, but falls far short of being sufficient to compensate for the rapid increase in the periphery of the cambium. Nägeli inferred from this that the elongated initials must divide periodically in a radial longitudinal plane. He even elaborated formulas for computing the frequency of such divisions during a given increase in the radius of a stem. However, as is not infrequently the case with *a priori* mathematical deductions concerning complex biological phenomena, Nägeli's generalization is supported by few, if any, of the actual facts. Although the hypothetical radial longitudinal divisions are described and figured in many botanical text-books, I have been unable to find them in any of the gymnosperms and less highly specialized dicotyledons that I have studied. The cells slowly elongate, sliding by one another, until they have reached a certain size. They then divide by means of a more or less oblique transverse partition into two short halves, which in turn elongate and divide.[2] Thus, the increase in the periphery of the cambium is due primarily not to radial longitudinal divisions of the large initials accompanied by lateral enlargement of the products of such divisions, but to the formation of transverse partitions, followed by elongation or longitudinal "sliding growth."[3]

[2] It should be kept clearly in mind, in this connection, that during the process of elongation, between successive transverse divisions, the cells continue to divide in the tangential longitudinal plane.

[3] This phenomenon of sliding growth is of considerable practical significance in the study of the properties and utilization of timber.

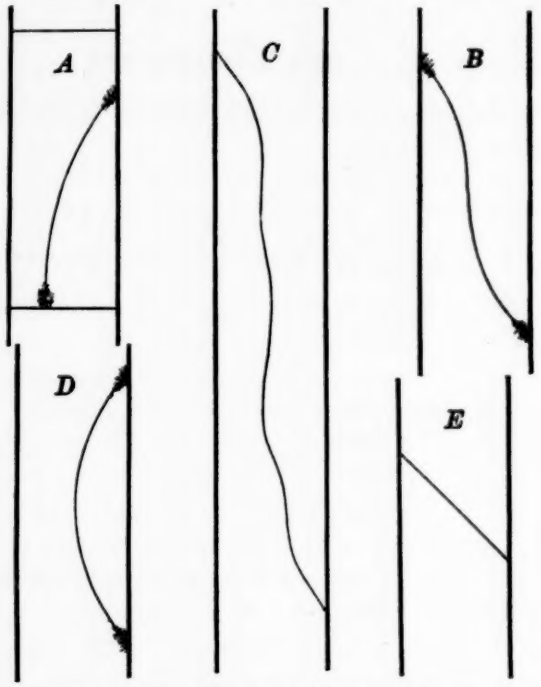


TEXT-FIG. 1. Frequency curves showing variation in the size of cambial initials in stems of different ages.



TEXT-FIG. 2. A, curve showing variations in the average size of large cambial initials with increasing age of a stem. B, size level of cells of the general order of magnitude of the cells of the embryo and growing points. E and E′, curves showing effects of experimentally induced changes in cell size.

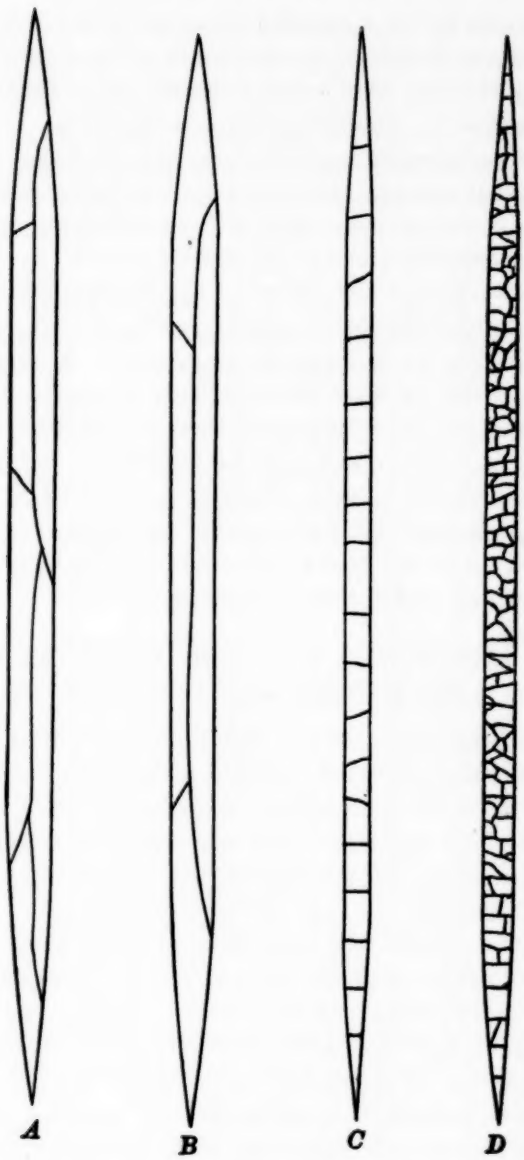
Owing to the fact that the initials do not divide and elongate in unison, there is usually a very considerable variability in the length, and *pari passu* in the volume, of adjacent elements. As shown in Text-fig. 1, the average, maximum and minimum lengths of the cells are considerably less in very young, slender, than in old, robust stems and roots; but the values do not continue to increase throughout the



TEXT-FIG. 3. Acute intersections of division membranes. A, from initial dividing to form callus. B, C, and E, from initials dividing pseudotransversely. D, from initial dividing unequally to form new ray initials.

entire life of an individual. On the contrary, the curve of average length at successive ages is of the general type illustrated in Text-fig. 2 (A). There is a rapid increase in length for a period of years until a certain size is attained, which then remains constant during succeeding growth.[4] A similar, though much less striking, increase

[4] The curve for any particular level or radius of a tree usually fluctuates about this norm, owing to the effects of various environmental factors.



TEXT-FIG. 4. A and B, acute intersections of division membranes in the daughter cells of the cambium of certain dicotyledons. C and D, angles of intersection in initials which are dividing to form callus.

commonly occurs in the tangential diameter of the cells; but in the gymnosperms the length of the initials is so great, in proportion to their breadth, that the volume curve closely parallels that for the longitudinal dimension. Under normal conditions the volume of the more or less isodiametric ray initials is very much less than that of even the smallest elongated initials (Figs. 1 and 2) and is of the same general order of magnitude as that of the undifferentiated cells of the embryo and terminal meristems or growing points.

To maintain a proper ratio between the two types of initials, in a layer whose periphery is continually increasing in area, new aggregations of ray initials are periodically carved out of the large initials by appropriate divisions of these elements (Text-fig. 3, D). Furthermore, under abnormal environmental conditions, *e.g.* injuries, all the elongated initials in a given area may be induced to divide into small, more or less isodiametric cells (Text-fig. 4, C and D). During the subsequent growth of this region of the meristem, certain of these small cells elongate and finally regenerate initials of normal dimensions, as shown by dotted lines in Text-fig. 2, E and E′.

The Problem of the Working Sphere of the Nucleus and the Maximum Size of Undifferentiated Plant Cells.

Sachs and Strasburger almost simultaneously called attention to the fact that undifferentiated, actively dividing and growing cells of plants, such as occur in embryonic and meristematic tissue, are relatively minute, and concluded that this was undoubtedly due to the fact that the working sphere of the nucleus is very restricted. Strasburger found that, in the case of "embryonic" cells of the growing points of various plants, the ratio between the average diameters of the nuclei and of the cells is as 0.003–0.016 mm.: 0.005–0.024 mm., or as 2:3; and Sachs pointed out that, although plants vary enormously in their linear dimensions (0.001 to 100,000 mm.), there is not a proportional variation (0.001 to 0.05 mm.) in the size of their constituent cells. Both investigators emphasized the fact that, even in highly differentiated tissues, unusually large or much elongated protoplasts[5] tend to be multinucleate.

[5] Such as have been critically studied by Schmitz, Treub, Kallen, Johow, Haberlandt, Pirotta, Buscalioni, and many others.

In view of these and similar investigations, it might have been expected *a priori* that the large, elongated, undifferentiated cells of the lateral meristem or cambium, which in certain plants attain a length of more than 10,000 micra and a volume of 10,000,000 cubic micra, should contain more than one nucleus each. Such is not the case, however, in any of the somewhat extensive series of gymnosperms and angiosperms that I have studied. Nor do the initials contain abnormally elongated, giant nuclei, such as have been described and figured by Molisch for highly specialized tissue cells of certain monocotyledons. Each initial contains a single nucleus, which is centrally located and retains this position during the processes of growth and cytokinesis.

It is evident, accordingly, that there is a very much greater variability in the size of meristematic cells in plants than was suspected by Sachs or Strasburger, and that in elements of this type the nucleus may extend its "energizing" influence to a distance of several thousand instead of a few micra.[6]

The Relation between Cell Size, Nuclear Size, and Chromosomal Mass.

Strasburger's measurements led him to believe that there is a close correlation between cell size and nuclear size in the meristematic tissue of plants, a conclusion that was strongly supported by the experimental investigations of Gerassimow. The importance of this relation between cell size and nuclear size was further emphasized by Hertwig, and by Boveri, who endeavored to prove that "The size of the larval cells is a function of the quantity of chromatin which they contain, and the volume of the cell is in direct proportion to the number of chromosomes." The subsequent painstaking and detailed investigations of a number of zoologists have indicated that, although in general large cells tend to have larger nuclei than small cells, the nucleocytoplasmic-relation is not invariably a constant and self-regulating ratio. In many animals, it fluctuates within rather wide limits, not only as between different tissues, but even in embryonic

[6] Sachs considered that the conditions in large animal eggs strengthened rather than weakened his case, since these highly specialized, yolk-containing cells are "inactive" until "energized" by numerous nuclei.

cells during different stages in ontogeny and under different environmental conditions. Conklin has emphasized the fact that in many cases the size of the nucleus is determined by the volume of protoplasm in which it lies, rather than by the number of chromosomes.

On the botanical side, Gates, Gregory, Winkler, and Tupper and Bartlett have shown that a number of races of plants, which have the tetraploid, instead of the diploid, number of chromosomes, are composed of cells larger than those of normal varieties. Winkler's paper is a particularly suggestive one, not only owing to the interesting experimental methods used in his work, but to his general discussion of the relation between cell size and chromosomal number in plants. He reaches the following conclusions, as a result of his own observations and those of a number of other investigators. In embryonic somatic tissue, terminal and lateral meristems, the cells are of nearly uniform size, are roughly isodiametric, and under normal conditions contain the diploid number of chromosomes. Multinucleate protoplasts, nuclear fusions, and changes from the diploid to the tetraploid and polyploid condition are of common occurrence in non-meristematic somatic tissue. In the latter tissue many cells depart widely from the inherited specific cell size of the plant. Such cells tend to be hyperchromatic; much elongated elements containing more than one nucleus each, and other types of large cells an abnormal number (tetraploid or polyploid) of chromosomes. In other words, Winkler considers that there is a very significant correlation between cell size and chromosomal mass, both in the embryonic and non-meristematic somatic tissues of plants.

As I have suggested at the beginning of this paper, the cambium appears to possess certain distinct advantages as a medium for studying various phases of the nucleocytoplasmic-relation. In this tissue adjacent cells vary greatly in shape and size and it is possible not only to compare elongated initials of very different lengths and volumes, but also to contrast them with adjoining ray initials which are of the same general order of magnitude as the cells of the embryo and terminal meristems. By proper experimental methods the long initials may be induced to divide into smaller and smaller units, until more or less isodiametric cells, which resemble the ray initials, are formed. These

may subsequently elongate and regenerate elements of normal size. In regions having cold winters, there is a more or less prolonged resting period, during which the cambial cells are inactive (not undergoing division). This period offers a favorable opportunity for measuring and computing the relative sizes of the initials and their nuclei. Furthermore, during the active growing season, adjacent cells (ray initials and elongated initials of varying sizes) may be found in equivalent stages of karyokinesis and cytokinesis, which facilitates comparisons within the limits of a single section.

TABLE I.

Pinus strobus L.

Type.	Nucleus.				Cell.				Ratio between volume of nucleus and volume of cell.
	Dimensions.			Approximate volume.	Dimensions.			Approximate volume.	
	L	R	T		L	R	T		
	Cambium from 1 yr. old stem.								
	micra	*micra*	*micra*	*cu. micra*	*micra*	*micra*	*micra*	*cu. micra*	
Ray initials.....	10.8	8.7	6.5	350	22.9	17.8	13.8	5,000	1:14
Large "	63	3.2	5.8	1,000	870	4.3	16.1	60,000	1:60
	Cambium from 60 yr. old stem.								
Ray initials.....	12.4	12.5	9.9	850	24.8	26.6	17.0	10,000	1:12
Large "	82	5.9	8.9	3,500	4,000	6.2	42.4	1,000,000	1:286

Basis: dimensions of cells and nuclei are averages of 50 measurements.

In 1917, 1918, and 1919, the writer collected numerous specimens of the cambium of *Pinus strobus* L.—from different parts of the stem and root, from trees of different ages and sizes, and from varying environments—at frequent intervals during the resting and growing seasons. As shown in Table I and in Figs. 1 to 6, the larger initials of the common white pine tend to have larger nuclei, but the ratio between cell size and nuclear size fluctuates greatly in the case of the elongated initials. So far as I have been able to determine, however, all the nuclei, regardless of their size, contain approximately the diploid number (twenty-four) of chromosomes. The larger size of the elongated

initials is not associated with a tetraploid or polyploid condition. Nor is it a concomitant of a marked increase in the size of the individual chromosomes, for I have repeatedly found small ray initials in which the chromosomes were fully as long and thick as those of adjoining large, elongated initials. The staining reactions of the various types of initials indicate that the chromatic material is more concentrated in the smaller than in the larger nuclei except during certain stages of karyokinesis; and that the increase in the size of the nuclei is due primarily to an increase in the volume of achromatic substances. Although there is no constant and striking difference in the number and size of the chromosomes in the large and small initials, *the volume of the nucleoli is conspicuously greater* in the larger cells (Figs. 3, 4, 5, and 6). This increase is associated with a *corresponding increase in kinoplasma* during karyokinesis and cytokinesis.

My observations upon *Pinus strobus*, therefore, do not support Strasburger's and Winkler's conclusions in regard to the constancy of "specific" cell sizes and nuclear sizes in the meristems of plants and are in opposition to Winkler's assumption that giant cells are hyperchromatic. In lateral meristems there are relatively great variations in cell size without corresponding changes in the number of chromosomes. In other words, in dealing with the nucleocytoplasmic-relation it is essential to distinguish between (1) those cases in which there is a correlation between cell size, nuclear size, and chromosomal mass, and (2) those in which chromosomal number (chromosomal mass) *is constant and nuclear size and cellular size are variable.*

Karyokinesis and Cytokinesis.

In the gymnosperms, as illustrated by *Pinus strobus*, the polar axis of the division figure usually does not stand at right angles to the long axis of the protoplast, but is placed diagonally across the cell (Fig. 7). This position of the karyokinetic figure is not an artifact, *i.e.* due to displacement of an ordinary spindle, since the whole figure is asymmetrically developed in conformity with its diagonal position. The formation of a cell plate, starting from one of these obliquely placed spindles, is a truly remarkable cytological phenomenon. The spindle becomes greatly extended laterally by the addition of peripheral "fibers," and gradually assumes a more or less curved

form (Figs. 8 and 9). As more peripheral fibers are successively added, the remains of the original central fibers disappear (Fig. 9) from about the cell plate, leaving two separate aggregations of fibers that are connected by the first formed portion of the cell plate (Fig. 10). These aggregations of kinoplasmic fibers, which I have called *kinoplasmasomes*, have a very characteristic form and structure, both in gymnosperms and angiosperms. When the initials are dividing in the usual tangential longitudinal plane, the kinoplasmasomes extend across the cell—at right angles to the longitudinal axis—from one radial wall to the other (Fig. 12) and are located in the center of the protoplast, midway between its tangential surfaces (Fig. 10). In sectional view, they have a somewhat wedge-shaped outline, bluntly convex in front and tapering to a point at the rear along the cell plate (Fig. 10). They move in opposite directions towards the ends of the cell (Figs. 11 and 12). As they move forward the cell plate is extended until it eventually reaches the two ends of the cell, thus dividing the protoplast into two similar halves, each of which contains one of the daughter nuclei (*n*). The latter remain close together, near the center of the cell, during the process of cell plate formation, and I have been unable to demonstrate any visible connection between the daughter nuclei (*n*) and the kinoplasmasomes (*k*) or their constituent fibers. Not infrequently the distance traversed by the kinoplasmasomes, in passing from the vicinity of the daughter nuclei to the ends of the protoplast, may be from 500 to 5,000 micra. The two kinoplasmasomes usually, although not invariably, move forward at *equal rates*, so that at any given stage they appear to be equidistant from the starting point and daughter nuclei. Preliminary investigations indicate that the total time consumed in the process of cell plate formation is considerable, in all probability a matter of many hours rather than minutes.

In the normal, tangential longitudinal divisions of the large initials, the kinoplasmasomes, once they have curved into a position midway between the tangential walls of the cell, move in a straight line towards the ends of the protoplast; thus the division is a plane surface. This is frequently not true, however, in the case of the semitransverse divisions of the initials. The kinoplasmasomes meander more or less in certain cases and curved or undulating surfaces are formed in consequence (Text-fig. 3).

Dynamics of Cytokinesis.

The phenomena of karyokinesis and cytokinesis in the cambium are in direct contradiction to most generalizations concerning cell division. According to Sachs' Law, successive division planes should intersect at right angles; but in the cambium the successive longitudinal partitions are parallel. Hertwig's development of Sachs' Law hypothecates that the axis of the mitotic figures typically lies in the longest axis of the protoplasmic mass, and division therefore tends to cut this axis at a right angle; but in the cambium the axis of the mitotic figure is usually placed either at right angles to the long axis of the cell (angiosperms) or in a diagonal position (conifers, angiosperms). Errera's (Plateau's) Law of Minimal Area is based upon the assumption that the recently formed membranes of cells are semiliquid films which "tend to assume a form which would be assumed, under similar conditions, by a liquid film destitute of weight;" but in the cambium the partitions are commonly division planes of maximal area. Furthermore, according to this law, the division membranes of most plant cells[7] should intersect the sides of the cells at right angles. De Wildeman, Thompson, and others contend that even the occurrence of oblique divisions in elongated plant cells does not necessarily invalidate the minimal area hypotheses, provided these partitions are sigmoid and intersect the side walls at right angles.

It is significant, accordingly, that in the cambial initials—as also in their daughter cells—of gymnosperms and dicotyledons, many partitions are formed which intersect the older, rigid walls at angles of varying degrees of acuteness (Text-figs. 3 and 4). These acute angles are not due to sliding growth and displacement of protoplasts. The peculiar type of cell plate formation in the cambium facilitates the study of successive stages in the formation of the division membranes and it is evident that the kinoplasmasomes, cell plates, and new cell walls frequently intersect the sides of the cells at very acute angles (Text-fig. 3).

[7] The older walls of most cells of the higher plants are relatively thick and rigid.

These facts, as those cited by Chambers, raise the question whether in dealing with cytokinesis we actually are concerned with protoplasm in liquid or semiliquid phases.

In any case, this type of cytokinesis, in which the process of cell plate formation is so greatly extended—both as regards space and time—and so clearly dissociated from the usual phenomena of karyokinesis, promises upon further and more critical analysis, to be of some significance in any general discussion concerning the dynamics of cell division.

SUMMARY.

1. The adjacent, undifferentiated, uninucleated cells of the lateral meristem or cambium are of two distinct shapes and sizes: (1) small, more or less isodiametric initials which are of the same general order of magnitude as the cells of the terminal meristem and embryo; and (2) large, elongated initials which in certain cases may attain a length of more than 10,000 micra and a volume of 10,000,000 cubic micra. The large initials may be induced to divide to form small initials, and the latter to regenerate elongated cells of normal dimensions. Thus, the cambium affords an unusually favorable medium for the study of a number of fundamental physiological and cytological problems.

2. A study of the cambium reveals the fact that there is a very much greater variability in the size of meristematic cells in plants than was suspected by Sachs or Strasburger, and that the working sphere of the nucleus is by no means so restricted as assumed by these investigators.

3. Although the larger cambial initials of *Pinus strobus* tend to have larger nuclei, the nucleocytoplasmic-relation varies within wide limits and the diploid number of chromosomes is constant. The conditions in the cambium do not support Winkler's view that there is a close correlation between chromosomal number (chromosomal mass) and cell size in the somatic tissue of plants, and that giant cells are hyperchromatic.

4. The process of cell plate formation in the cambium is a remarkable phenomenon, and one which is significant in discussing the relative merits of various theories concerning the dynamics of karyokinesis and cytokinesis.

5. The newly formed partition membranes in the cambial initials frequently intersect the side walls at angles of varying degrees of acuteness, which is in contradiction to Errera's (Plateau's) Law of Minimal Area.

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EXPLANATION OF PLATE 1.

FIG. 1. Scale drawing, showing the relative sizes of ray initials and long initials in the cambium of a 60 year old stem of *Pinus strobus* L. × 100.

FIG. 2. Scale drawing, showing the relative sizes of ray initials and large initials in the cambium of a year old stem of *Pinus strobus*. × 100.

FIG. 3. Nucleus from large initial shown in Fig. 1. × 1,000.

FIG. 4. Nucleus from large initial shown in Fig. 2. × 1,000.

FIG. 5. Nucleus from ray initial shown in Fig. 1. × 1,000.

FIG. 6. Nucleus from ray initial shown in Fig. 2. × 1,000.

FIG. 7. Karyokinetic figure in longitudinally dividing cambial initial of *Pinus strobus*. × 3,000.

FIGS. 8, 9, and 10. Early stages in the formation of kinoplasmasomes and cell plate; (*n*) daughter nuclei, (*k*) kinoplasmasomes. × 3,000.

FIG. 11. Radial longitudinal extension of cambial initial, showing later stage in the formation of the cell plate. × 100.

FIG. 12. The same. Tangential longitudinal extension. × 100.

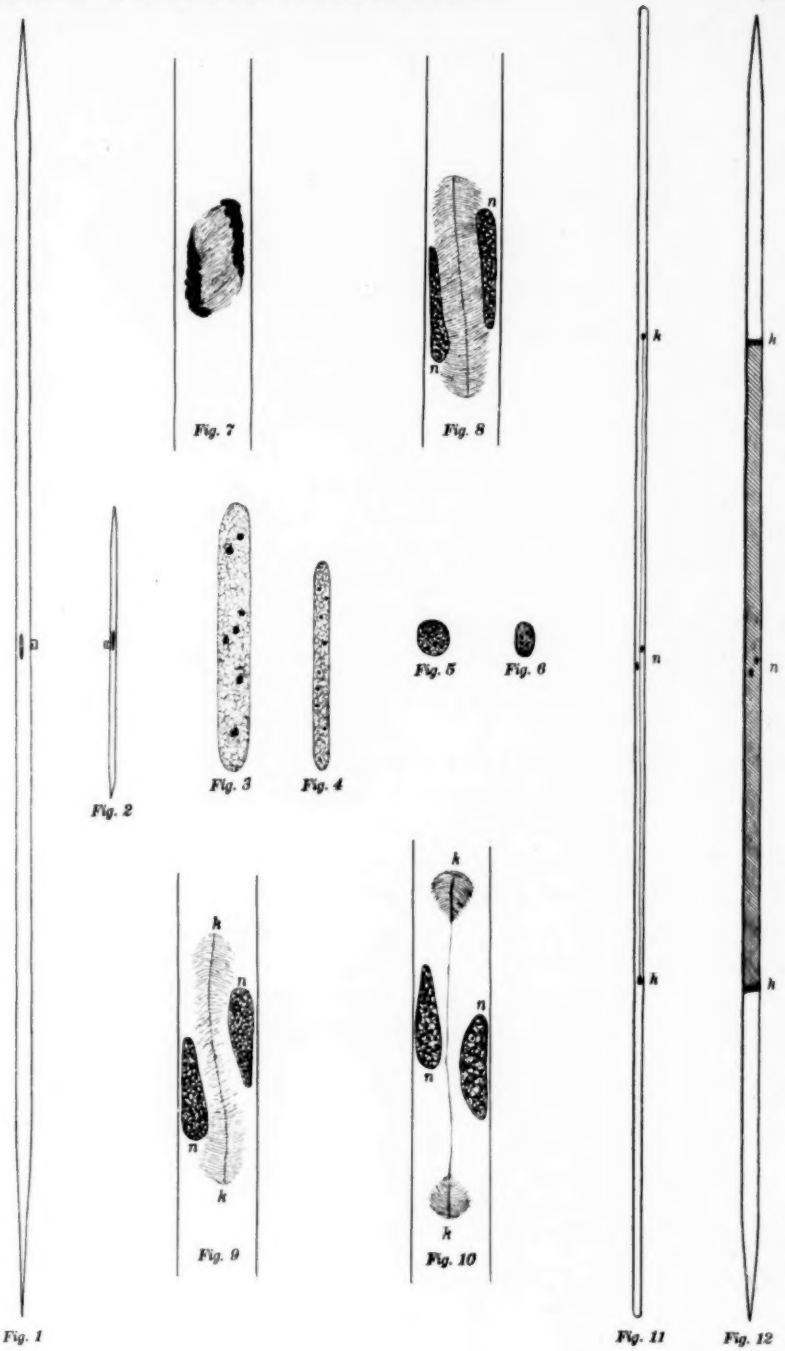

Fig. 1 Fig. 2 Fig. 3 Fig. 4 Fig. 5 Fig. 6 Fig. 7 Fig. 8 Fig. 9 Fig. 10 Fig. 11 Fig. 12

A QUANTITATIVE STUDY OF THE EFFECT OF ANIONS ON THE PERMEABILITY OF PLANT CELLS.

By ORAN L. RABER.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, January 25, 1920.)

Although the effects of cations on the permeability of plant cells have frequently been studied, comparatively little attention has been paid to the action of anions.

The writer has investigated the effects of a series of anions on the permeability of *Laminaria agardhii* Kjellm. Measurements were made of the electrical conductivity by the method described by Osterhout.[1]

The salts were the purest obtainable and the distilled water was not toxic to delicate test objects. The salt solutions were neutral to phenolsulfonephthalein (pH 7 ± 0.5). The temperature during the course of the experiments was 19 ± 2°C.

Sodium sulfocyanide, iodide, nitrate, bromide, chloride, acetate, sulfate, tartrate, phosphate, and citrate were selected for study.

Solutions of these salts were made of the same conductivity as a slightly diluted sea water solution; *viz.*, a solution having the same conductivity as a 0.51 M solution of sodium iodide. (Normal sea water has the same conductivity as a 0.57 M solution of sodium iodide.) As is well known, various samples of sea water differ slightly in their conductivity and hence no absolute concentration can be given for each salt. The following table shows the approximate concentrations used.

Salt	SCN	I	NO_3	Br	Cl	Acetate.	SO_4	Tartrate.	PO_4*	Citrate.
M	0.62	0.51	0.59	0.51	0.45	1.1	0.36	0.59	0.35	0.43

*The phosphate is a mixture of 0.23 M disodium phosphate and 0.12 M monosodium phosphate.

[1] Osterhout, W. J. V., *J. Biol. Chem.*, 1918, xxxvi, 557.

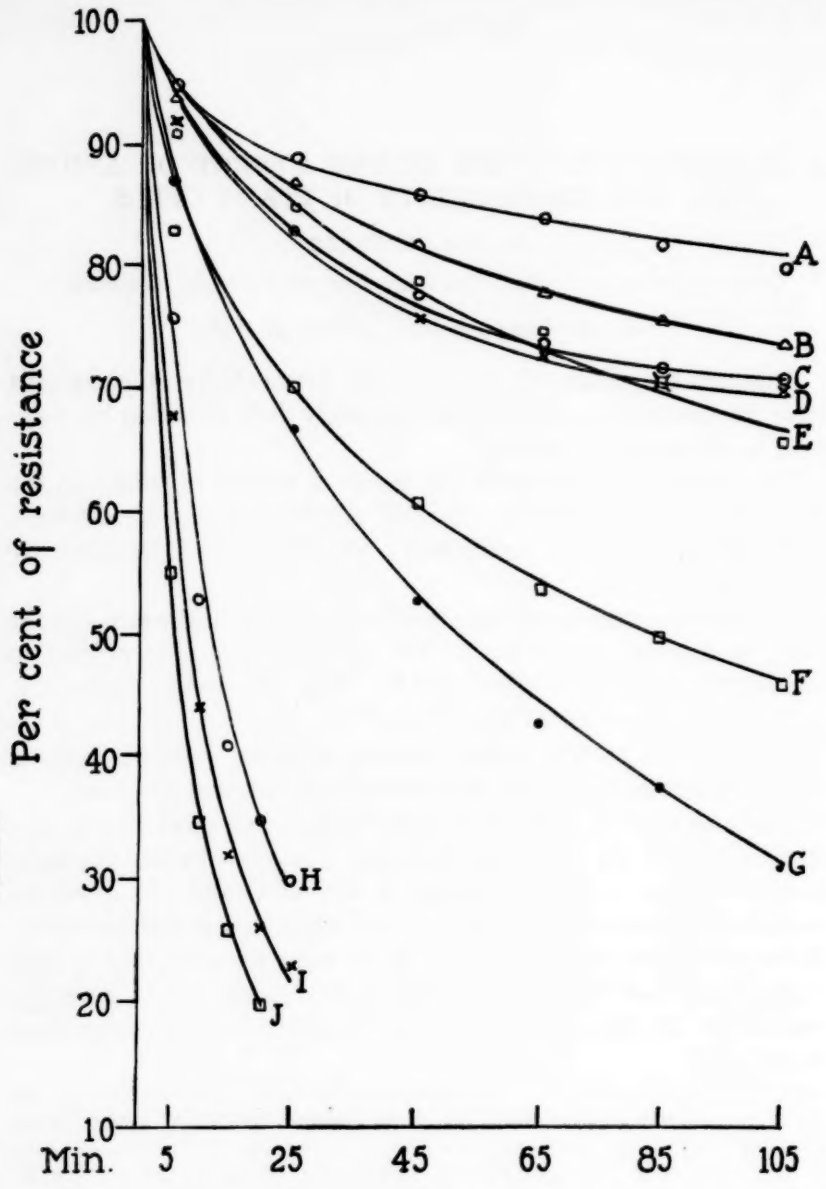

FIG. 1. Curves showing the resistance of *Laminaria* in solutions of various sodium salts: A in iodide; B in bromide; C in sulfocyanide; D in chloride; E in nitrate; F in acetate; G in sulfate; H in tartrate; I in phosphate; J in citrate. Ordinates represent resistances (expressed as per cent of the resistance in sea water, which is taken as 100 per cent). Each point represents the average of ten experiments. Probable error less than 10 per cent of the mean.

Measurements of the conductivity (permeability) were taken at the end of 5, 25, 45, 65, 85, and 105 minutes in the salt solution except in certain solutions (citrate, phosphate, and tartrate) where the resistance diminished very rapidly.

The results are shown in Fig. 1, the curve for each salt representing an average of ten experiments. The probable error of the mean (as based on Peter's formula) is always under 10 per cent, and for 75 per cent of the points is under 5 per cent.

It will be noted that the effect of these anions upon the increase in permeability as seen at the end of the experiment is in the series I, Br, SCN, Cl, NO_3, acetate, SO_4, tartrate, PO_4, citrate.

This differs from the Hofmeister series in that the effect of SCN is noticeably more than that of the iodide while the two effects were indistinguishable by Hofmeister, and also in that the relative positions of chloride and nitrate are reversed in this work from that found by both Pauli and Hofmeister. That these positions, however, are not invariable has been shown by Höber who obtained so called "transition series" by varying the hydrogen ion concentration[2] and the concentration of the salt.[3] It may be noted, however, that at the end of the 25, 45, and 65 minute periods, when the disturbance affecting the experiment at the start has subsided and before possible disturbing factors due to the purely mechanical deterioration of the tissue have appeared, the position of the nitrate is above that of the chloride.

It is also seen that there is a distinct grouping into two or three groups. The iodide, bromide, sulfocyanide, chloride, and nitrate behave very similarly, while the acetate, sulfate, tartrate, phosphate, and citrate have a much more striking effect. In order to have solutions of the same conductivity as the sea water used, it was found necessary, as already mentioned, to employ different concentrations. But it is also desirable to make a comparison on the basis of the same molecular concentration. Since the concentration of two of the salts was close to 0.52 M and since this is the concentration of sodium

[2] Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 4th edition, 1914, 323.

[3] Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 4th edition, 1914, 324.

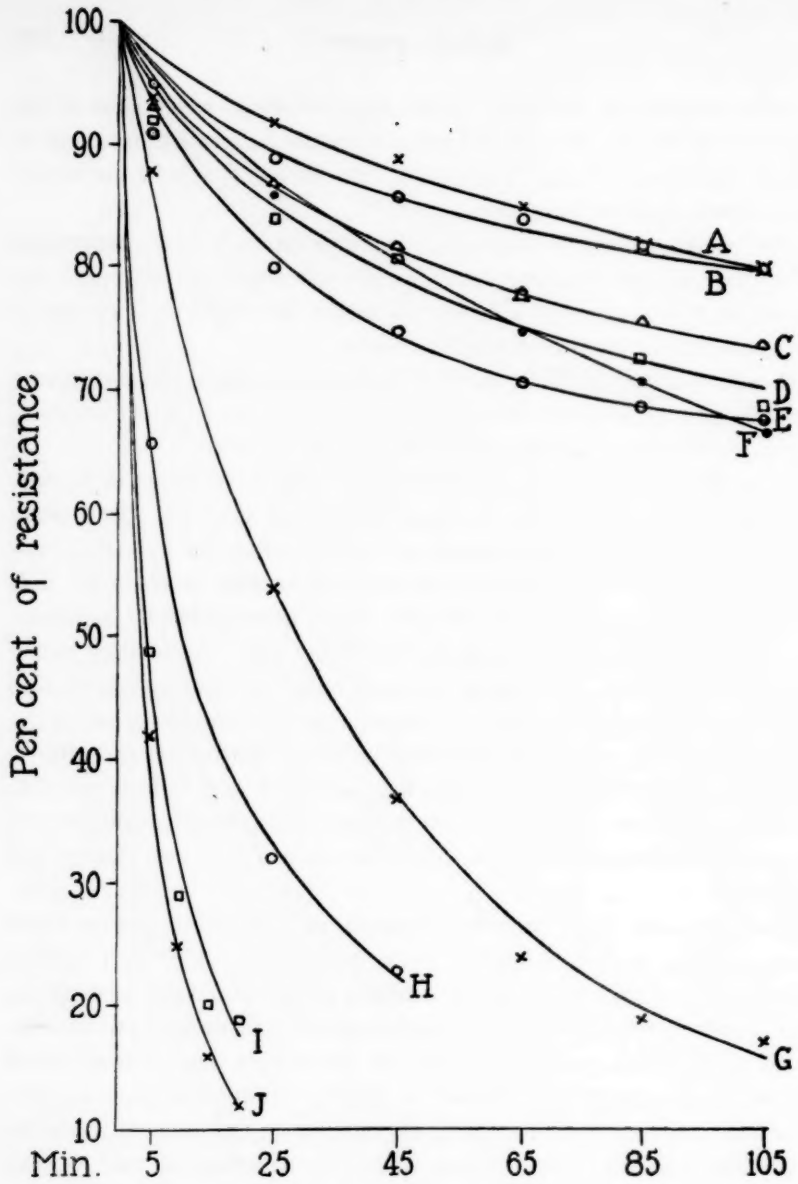

FIG. 2. Curves showing the resistance of *Laminaria* in solutions of various sodium salts: A in sulfocyanide; B in iodide; C in bromide; D in nitrate; E in chloride; F in acetate; G in sulfate; H in tartrate; I in phosphate; J in citrate. Ordinates represent resistance (expressed as per cent of the resistance in sea water, which is taken as 100 per cent). Each point represents the average of ten experiments. Probable error less than 10 per cent of the mean.

chloride required to produce a solution of the same average conductivity as undiluted sea water, this was the molecular strength decided upon.

Sea water was concentrated (or diluted) in each case until it possessed the same conductivity as the solution to be tested. After the tissue had been placed in the concentrated (or diluted) sea water long enough to come to equilibrium (20 to 30 minutes) a measurement of the resistance was taken and the material then placed in the salt solution after which the procedure was the same as in the previous experiments.

Fig. 2 shows the results of these experiments. The results for each concentration of each salt are the average of ten experiments as before, all the concentrations being 0.52 M, with the exception of that of the phosphate. In this case the solubility of the disodium salt prohibits a concentration higher than 0.26 M; enough of the monosodium salt was added to produce neutrality and the total molecular concentration reckoned on this basis was about 0.39 M.

In this series the sulfocyanide takes its place at the top along with the iodide, and at the end of the experiment the two were identical. The nitrate curve is raised a trifle so that it now comes between the bromide and the chloride. The chloride, phosphate, and citrate drop slightly and the tartrate rises slightly, all of which changes are to be expected.

The greatest changes occur in the sulfate and acetate, which were farthest from 0.52 M in the first set of experiments. The acetate rises and takes its place among the other monovalent salts and the sulfate drops towards the tartrate and the trivalent salts, thus forming two distinct groups. It should be noted that the acetate comes from Group II up to Group I, but that its rate of fall at the end of the experiment was greater than for the others.

Several explanations for these phenomena may be given, but it does not seem worth while to enter upon a discussion of them at present. The recent work of Loeb on the relation between gelatin and electrolytes[4] may furnish a clue to the ultimate explanation.

[4] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 39, 237, 363, 483, 559.

THE ANTAGONISTIC ACTION OF ANIONS.

By ORAN L. RABER.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, February 10, 1920.)

Striking cases of antagonism among cations are well known but the search for similar relations among anions has achieved little result. Some cases have been described by Loeb[1] and Miss Moore.[2] Lipman and his associates[3] have reported antagonistic action of anions as the result of studies on bacteria in which salts were added to the soil, but it is very difficult to separate the effects of the added salts from those already present in the soil. Miyake[4] found some antagonism among anions in studying the growth of wheat. Fenn[5] has called attention to the fact that this kind of antagonism is commonly met with in experiments on gelatin.

The writer has observed some cases of this kind of antagonism among plants. A quantitative study of one of these cases is here presented. This study was carried on at Cambridge in the fall of 1919. The marine alga, *Laminaria agardhii* Kjellm was employed, the material being shipped from Woods Hole to Cambridge as needed. The material when it arrives in the laboratory is known to be in good condition since its resistance can be measured and compared with that of freshly gathered material. When kept at a low temperature the alga usually maintains its original resistance and vitality for several days. It was usually shipped in a covered firkin containing sea water. The firkin was surrounded by ice and was water-tight so that water from the melting ice could not dilute the sea water.

[1] Loeb, J., *Arch. ges. Physiol.*, 1905, cvii, 252 and literature cited there.

[2] Moore, A., *Am. J. Physiol.*, 1901, v, 87; 1902, vii, 315.

[3] Lipman, C. B., *Centr. Bakteriol., 2te Abt.*, 1912-13, xxxvi, 382. Lipman, C. B., and Burgess, P. S., *Centr. Bakteriol., 2te Abt.*, 1914, xli, 430; 1914–15, xlii, 502. Lipman, C. B., *Plant World*, 1914, xvii, 295.

[4] Miyake, K., *J. Coll. Agric. Tohoku Imp. Univ.*, 1914, v, 241; *J. Biol. Chem.*, 1913-14, xvi, 235.

[5] Fenn, W. O., *J. Biol. Chem.*, 1918, xxxiv, 141.

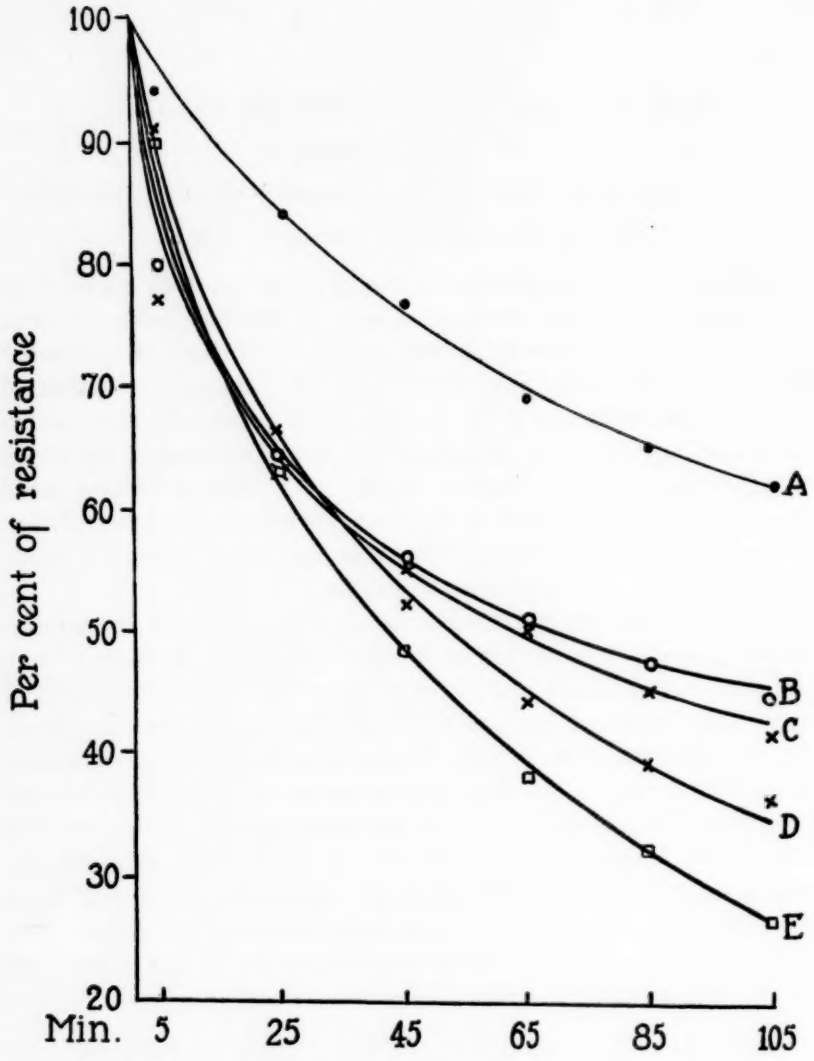

FIG. 1. Curves showing the resistance of *Laminaria* in 1.1 M sodium acetate, in 0.36 M sodium sulfate, and in mixtures of both: A in equal parts (by volume) of acetate and sulfate; B in acetate 75, sulfate 25; C in acetate; D in acetate 25, sulfate 75; E in sulfate. Ordinates represent resistance (expressed as per cent of the original resistance in sea water which is taken as 100 per cent). Each point represents the average of ten experiments: probable error of the mean less than 5 per cent.

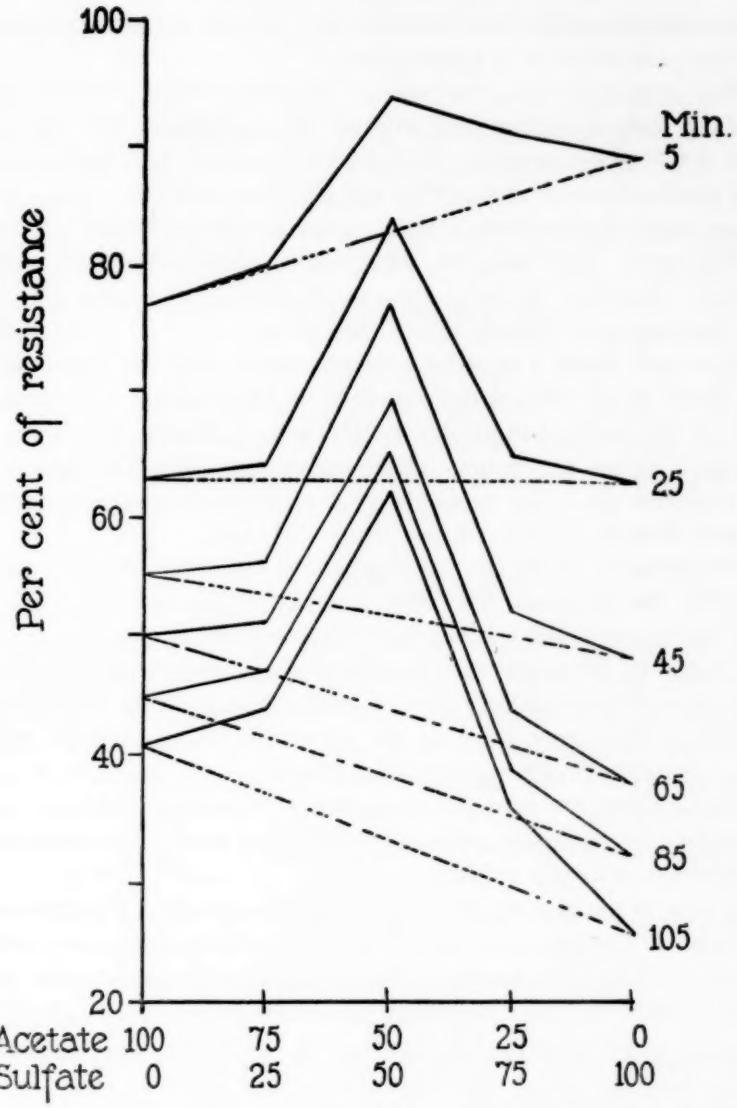

FIG. 2. Antagonism curves showing the resistance of *Laminaria* in 1.1 M sodium acetate, in 0.36 M sodium sulfate, and in mixtures of both. Ordinates represent resistance (expressed as per cent of the original resistance in sea water which is taken as 100 per cent); abscissæ represent volumetric proportions of the two salts. The dotted line connecting the ends of each curve shows the approximate additive effect; the vertical distance of the curve above this dotted line may be regarded as a measure of antagonism.

The method employed to measure the effects of the salts is the same as that mentioned in a former paper.[6]

The salts used were the purest obtainable. The distilled water was not toxic to delicate test objects. The solutions had a pH value of 7 ± 0.5 as measured by phenolsulfonephthalein and buffers which had previously been checked by the gas chain method. The experiments were conducted at a temperature of 19 ± 2°C.

This paper deals with the effects of sodium acetate and sodium sulfate. Solutions of these salts were made of the same electrical conductivity as a slightly diluted sea water (about 1.1 M for sodium acetate and about 0.36 M for sodium sulfate) and the resistance of the tissue in solutions of the pure salts and in mixtures was measured after 5 minutes and thereafter at intervals of 20 minutes.

Fig. 1 shows the results. Each curve represents the average of ten experiments. The probable error of the mean (as computed by Peter's formula) is under 5 per cent in all cases.

On placing tissue in the pure acetate we observe that at the end of $1\frac{3}{4}$ hours the resistance has fallen to about 40 per cent of the original and in the pure sulfate it has fallen to about 25 per cent of the original, while in the mixture composed of equal volumes of the solution of each salt the resistance has fallen only to about 60 per cent. If no antagonism were present, the resistance of the mixture should drop to about 35 per cent (additive effect).

Fig. 2 shows the antagonism curves after various intervals using resistance for ordinates and salt proportions as abscissæ. Here the antagonism is clearly evident.

In view of the importance of the subject, further investigation seems desirable. The case here described is so striking as to warrant the conclusion that any theory of the antagonistic action of salts must take account of anions as well as of cations.

[6] Raber, O. L., *Proc. Nat. Acad. Sc.*, 1917, iii, 682.

THE NATURE OF THE GROWTH RATE.*

By H. S. REED.

(From the University of California Graduate School of Tropical Agriculture and Citrus Experiment Station, Riverside.)

(Received for publication, March 5, 1920.)

Recent studies (Robertson (1908) and Reed and Holland) have shown that the rate of growth of certain organisms corresponds to the rate of an autocatalytic reaction. At the present time it seems profitable to extend the inquiry to several different types of plants and to learn something of their growth rates.

The Growth Rate of Pear Shoots.

During the season of 1919, weekly measurements were made of the growth of shoots of young Bartlett pear trees. The young shoots of the pear (*Pyrus communis* L.) are good material for such inquiry because of their vigorous growth and generally unbranched condition during the 1st year. The trees on which the shoots were selected and marked had been planted 3 years previously. They made good growth during the season in which the measurements were being made, but, on account of their age, produced no fruit. On April 24, 1919, fifty shoots on twenty-six trees in different parts of the orchard were selected. One shoot was subsequently broken and had to be eliminated. The remaining forty-nine shoots were measured each week until September 3, at which time elongation had ceased. The length of each new shoot was determined weekly by measuring the distance from its base to the apical bud. Though a few shoots did not cease elongation until the 133rd day (September 3) the average length of the growing season of all shoots was 97 ± 1.7 days.

* Contribution No. 65 from the University of California Graduate School of Tropical Agriculture and Citrus Experiment Station.

Robertson has called attention to the correspondence between growth of organisms and autocatalysis. He has shown that the rate of growth may also be expressed by the differential equation

$$\frac{dx}{dt} = kx\,(a - x)$$

in which x is the size of the organism at time t; a is the final size of the organism; and k is a constant. When integrated, the above equation becomes

$$\log \frac{x}{a - x} = K(t - t_1)$$

where $K = ak$, t_1 is the time at which the organism has reached half its final size; *i.e.*, the time when $x = \frac{a}{2}$. With the assistance of tables which Robertson (1910–15) has prepared, the constants and the theoretical values of x are easily obtained from observational data. The value of a was taken as 114 cm. and t_1 as 47.4 days. Table I shows the computations made and the several values of K. With the average value of K the values of x were calculated by the formula and are given in the fourth column of Table I. The agreement between the two is good except for the first three measurements. Fig. 1 shows the curve obtained from the calculated values together with the observed values of mean length at weekly intervals during the growing season.

This growth rate is of considerable physiological interest if we compare it with the growth rates of organisms mentioned elsewhere in the literature. Attention may be called to the fact that we are here dealing with the mean growth of a number of separate shoots on a small population of trees.

A word concerning the heterogeneity of the population may be useful. On the final day of measurements the shoots ranged in length from 69 to 171 cm. with an average of 113 cm. The standard deviation of the measurements was 21.14 cm. This indicates that we are dealing with a random selection of material and not one which was so closely selected that it was not representative of the shoots of such trees.

TABLE I.

Growth Rate of Bartlett Pear Shoots for the Season of 1919.

t	x (observed).	K	x (calculated).
days	*cm.*		*cm.*
0	0		8.1
7	5	0.0331	10.7
14	11	0.0292	15.4
21	19	0.0264	21.3
28	29	0.0241	28.8
35	40	0.0215	38.1
42	48	0.0256	48.5
49	59	0.0194	54.4
55	69	0.0243	69.0
63	76	0.0194	80.4
70	86	0.0215	88.8
77	94	0.0228	95.6
84	100	0.0233	100.9
91	102	0.0214	104.8
98	108	0.0247	107.6
105	110	0.0250	109.6
112	111	0.0244	110.9
119	112	0.0243	111.9
126	113	0.0260	112.6
Average		0.0242	

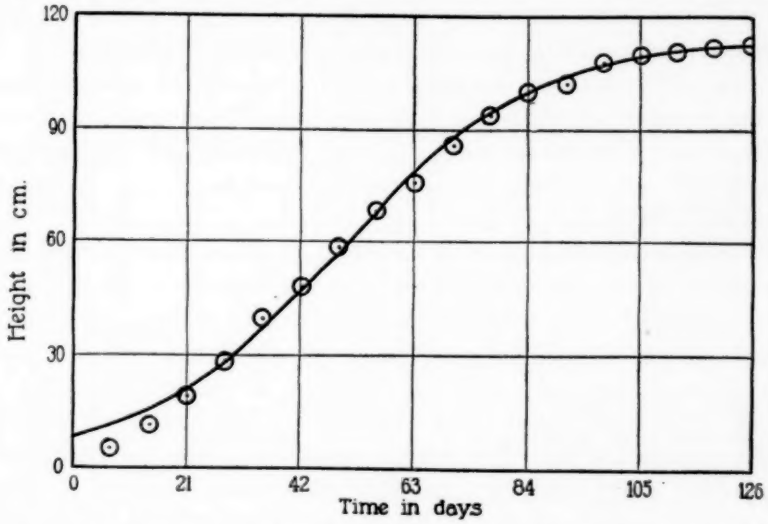

FIG. 1. Growth rate of shoots of the pear tree. The curve represents the value obtained from the equation $\log \frac{x}{114-x} = K(t-47.4)$; points within the circles represent observed lengths at intervals of 7 days.

The data suggest that each shoot has a more or less independent supply of the catalytic agent which exercises such an important influence upon the rate at which growth proceeds.

The Growth Rate of Young Walnut Trees.

The data upon which this study is based were obtained from material growing in the grounds of the Citrus Experiment Station at Riverside.

In the spring of 1916 seeds of *Juglans nigra* L. (black walnut) had been planted in rows for nursery propagation work. The young trees which grew from these seeds were used for study in the summer of 1917; *i.e.*, during their second season of growth. A part of the trees were grafted in the spring of 1917 with scions of *Juglans regia* (Persian walnut). A few weeks after growth from the scions started, all new shoots were removed, except one which furnished the data for the observations on this species. The balance of the *Juglans nigra* trees grew on during the second summer.

The *Juglans nigra* seedlings were selected on April 17 and marked by a line of India ink on the stem from which the distance to the terminal bud could be measured when desired. From a portion of the trees all laterals on the wood of the previous year were removed before measurements were begun, and the observations on these pruned trees were separately recorded. The growth from the *Juglans regia* scions was soon sufficient to afford measurements, and they were marked on May 29.

Measurements were made at each day of observation to determine the height of the tree stem from the ink mark to the terminal bud of the main axis. This could be ascertained with comparatively little error. It was planned to make measurements at intervals of 7 days, but other circumstances made it necessary to vary this interval at times. The exact length of the intervals is stated in Table II.

The population thus gave an opportunity for studying the following points: (*a*) the growth of seedling trees in the 2nd year of their life; (*b*) the growth of similar trees from which all laterals had been removed at the beginning of the 2nd year; and (*c*) the growth from scions of a distinct species growing on the same roots as (*a*) and (*b*).

Group (*a*) contained nineteen trees, Group (*b*) fifteen trees, and Group (*c*) twenty-five trees.

The mean height of the selected trees on the several days of measurement is represented graphically in Fig. 2. The data show that *Juglans nigra* grew quite uniformly from the commencement of the measure-

TABLE II.

Mean Height of Walnut Trees.

Days from Apr. 17.	Mean height of *Juglans nigra* trees. Laterals not removed.	Mean height of *Juglans regia* trees on *Juglans nigra* roots.
	cm.	*cm.*
0	–	–
7	56.2 ± 1.92	
14	61.2 ± 1.73	
21–	64.4 ± 2.37	
28	69.1 ± 2.68	
34	71.5 ± 2.71	
41	73.5 ± 2.82	
48	77.0 ± 2.99	
55	79.1 ± 3.03	63.24 ± 0.55
62	80.9 ± 3.14	67.80 ± 0.66
69	84.6 ± 3.23	74.88 ± 0.95
84	105.9 ± 3.23	102.6 ± 2.46
87	109.6 ± 3.70	110.2 ± 2.65
96	117.6 ± 4.01	126.6 ± 3.47
103	127.6 ± 4.55	140.6 ± 3.97
110	130.3 ± 5.01	152.0 ± 4.25
117	131.8 ± 5.25	162.4 ± 4.78
131	132.9 ± 5.15	176.0 ± 5.30
138	134.5 ± 5.65	184.0 ± 5.76
144		188.8 ± 5.82
151		195.2 ± 6.35
158		198.4 ± 6.47
165		199.2 ± 6.67
172		200.0 ± 6.87
179		200.8 ± 6.80

ments on April 17 until about June 19 (the 62nd day). Between June 19 and August 6 (between the 62nd and 110th days) growth was considerably more rapid, but after August 6 growth gradually diminished until it ceased about September 3.

The mean growing period of this group of trees was 124 days after April 17. The coefficient of variability of the population at each

time of measurement had an approximate value of 25. There are fluctuations from this value, but the trees showed little tendency to increase in variability until near the end of the growing period.

The *Juglans nigra* trees from which all laterals were removed in the spring before growth began made the same type of growth as their unpruned neighbors, but in every case their mean height was less. The commonly accepted belief is to the effect that removing the lat-

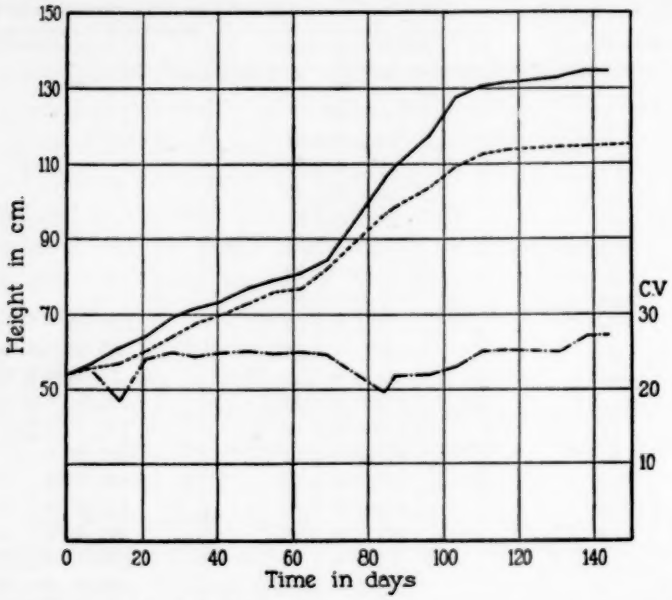

FIG. 2. The growth of *Juglans nigra* trees. ——— increase in mean height of unpruned trees. - - - - - increase in mean height of trees from which laterals had been removed. — . — . — Coefficient of variability of mean height of unpruned trees.

eral branches from a plant increases the growth of the main axis, but the results of this work directly contradict the above mentioned belief.

The mean growing period of the pruned seedlings was 122 days, or practically the same as that of the unpruned seedlings. Comparison of the figures shows that the pruned seedlings increasingly lagged behind their unpruned neighbors as the season advanced.

The *Juglans regia* trees were measured from June 5 to October 22. The data afforded by these measurements are shown in Table II. The average length of their growing season was 116 days from June 5, which means that the average tree ceased to grow on September 28, though it was October 22 before all trees ceased to increase in height.

We may proceed to examine the growth rate of these trees by a method which has elucidated many problems in the domain of physical chemistry.

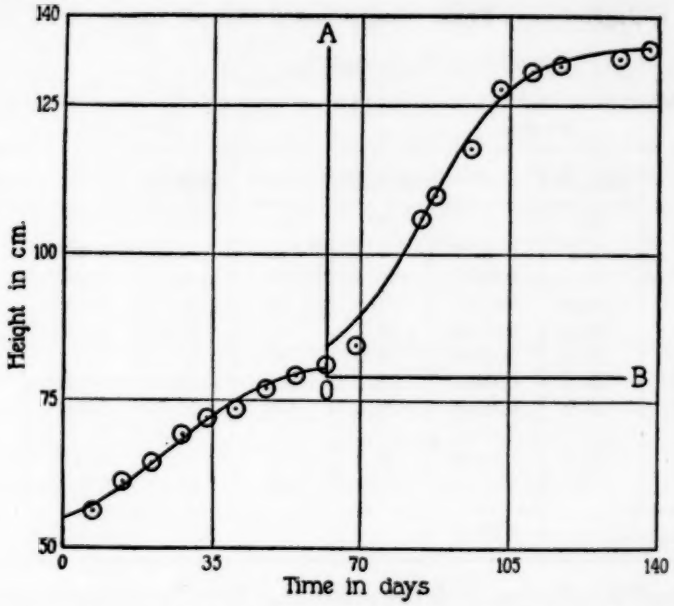

FIG. 3. Growth rate of *Juglans nigra* trees showing two cycles. Curves show computed values; points within circles show observed mean height at various intervals. AOB are axes for the upper curve.

Reference to the graph representing the mean height of the trees at successive intervals shows that the curve consists of two rather distinct parts, indicating that the growth period comprised in these records consisted of two cycles. The curve rises rather regularly from the 1st to the 62nd day, then rises more steeply to near the 110th day, when it takes a nearly horizontal position. A few simple calculations will show that the growth curve does not follow the path of a single autocatalytic reaction.

The period was therefore divided into two cycles, the first of which ended at the 62nd day, and the second of which began at approximately the time the first cycle ended. Reference to Table III will show the computations made. Since we are interested in rate it will be proper to move our base line up to a point near that at which the first cycle actually started. Actually 50 cm. were subtracted from each mean in order to give a curve which would start near the inter-

TABLE III.

Height of Juglans nigra Trees. Comparison of Observed Values with Those Computed from the Formula $\log \frac{x}{a-x} = K(t-t_1)$.

First cycle.				Second cycle.			
t	$x-50$ (observed).	K	$x-50$ (calculated).	t	$x-79$ (observed).	K	$x-79$ (calculated).
days	*cm.*		*cm.*	*days*	*cm.*		*cm.*
7	6.2	0.0375	7.2	62	1.9	0.0632	5.4
14	11.2	0.0283	10.5	69	5.6	0.0596	9.7
21	14.4	0.0348	14.5	84	26.9	0.0350	26.7
28	19.1	0.0380	18.7	87	30.6	0.0400	30.7
34	21.5	0.0296	22.0	96	38.6	0.0313	41.7
41	23.5	0.0252	25.2	103	48.6	0.0454	47.7
48	27.0	0.0299	27.6	110	51.3	0.0415	51.5
55	29.1	0.0317	29.2	117	52.8	0.0381	53.6
62	30.9	0.0374	30.3	131	53.9	0.0308	55.4
				138	55.5	0.0385	55.7
Average		0.0325	.....	.....		0.0423	

section of the abscissa with the ordinate. For the first cycle the value of a was taken as 32, and t_1 as 23.5. Accordingly

$$\log \frac{x}{32-x} = K(t-23.5)$$

From this, the several values of K were computed and found to be fairly uniform. The average value of K was taken as 0.0325, and from it the values of x were computed by the above formula. The root-mean-square deviation $\left(\sqrt{\frac{d^2}{n}}\right)$ from the observed values was only 0.78 cm. It will thus be seen that the agreement between observed and calculated values is good.

The data for the second cycle were similarly treated. The values were all diminished by 79 cm. The value of a was taken as 56 and that of t_1 as 85. The mean value of K was determined to be 0.0423 and from this the values of $x-79$ were calculated by the same general formula as for the other cycle. The agreement between the calculated and observed values is quite good. A graphical representation of the values is shown in Fig. 3.

It thus appears that between April 17 and September 4 the growth of this population of *Juglans nigra* trees fell into two distinct cycles and that the rate of growth for each cycle followed closely the course of an autocatalytic reaction.

A similar type of growth rate was also found for the *Juglans nigra* trees from which the laterals had been removed at the beginning of the season. It has been shown (Reed and Holland) that the smaller members of a population have a type of growth rate similar to that of the larger individuals. That is, both groups were found to correspond to the equation which states that the growth in unit time is proportional to the product of the height at that time and the amount yet to grow, multiplied by a constant.

The growth rate of the *Juglans regia* trees was likewise studied. Here again it is advisable to deduct a constant amount from each height measurement in order to bring the base line near the point of origin of the part of the growth curve studied. In this case $x_1=x-50$. The value of a was found by approximations to be 153.2 cm. and $t_1=96$. We therefore have

$$\log \frac{x}{153.2-x_1} = K(t-96)$$

Table IV shows the observed values of x_1, the various values of K, and the calculated values of x_1 with the mean value of K. The root-mean-square deviation is 3.5 cm. The smallness of this divergence becomes apparent when we note that the probable errors of the mean values of x (Table III) are greater than 3.5 except in the first six cases. A graphic representation is given in Fig. 4.

It will be noted that the data for the growth rate of *Juglans regia* extend from June 5 to October 15. There appears to be but one growth cycle between these dates. This period begins close to the

time at which *Juglans nigra* began its second growth cycle. We do not know whether *Juglans regia* had two growth cycles, but the presumption is in favor of such an hypothesis.

Data such as these afford a new insight into certain phases of growth in the organic world. They show that the growth rate of these or-

TABLE IV.

Height of Juglans regia Trees. Comparison of Observed Values with Those Calculated from the Formula $\log \frac{x}{a-x} = K\ (t-t_1)$.

t	x_1 (observed).	K	x (calculated).
days	*cm.*		*cm.*
55	13.2	0.0250	15.9
62	17.8	0.0261	22.1
69	24.9	0.0264	29.9
84	52.6	0.0233	53.2
87	60.2	0.0210	58.8
96	76.6		76.6
103	90.6	0.0229	90.5
110	102.0	0.0213	103.6
117	112.4	0.0210	115.1
131	126.0	0.0190	134.7
138	134.0	0.0201	138.0
144	138.8	0.0205	141.9
151	145.2	0.0229	145.1
158	148.4	0.0244	147.5
165	149.2	0.0228	149.2
172	150.0	0.0222	150.4
179	150.8	0.0216	151.2
Average		0.0228	

ganisms follows a course as definite and as predictable as that of a chemical reaction. On the basis of pure chance we should not expect often to find a group of individuals which should follow a definite rate of development more closely than the laws of random sampling would require.

The onset of dormancy in the two species of *Juglans* is worthy of notice. It should be remembered that the two lots of trees grew in adjoining rows in the nursery and received similar care as to irrigation,

cultivation, etc. Nevertheless the *Juglans regia* trees grew 42 days later than the *Juglans nigra* trees. It is well known that there is a great difference between these species in respect to distribution and resistance to cold weather. *Juglans regia* is reported to be a native of Persia, the Himalayas, and China, while *Juglans nigra* is a native of the eastern United States. The remarkable fact, as far as these

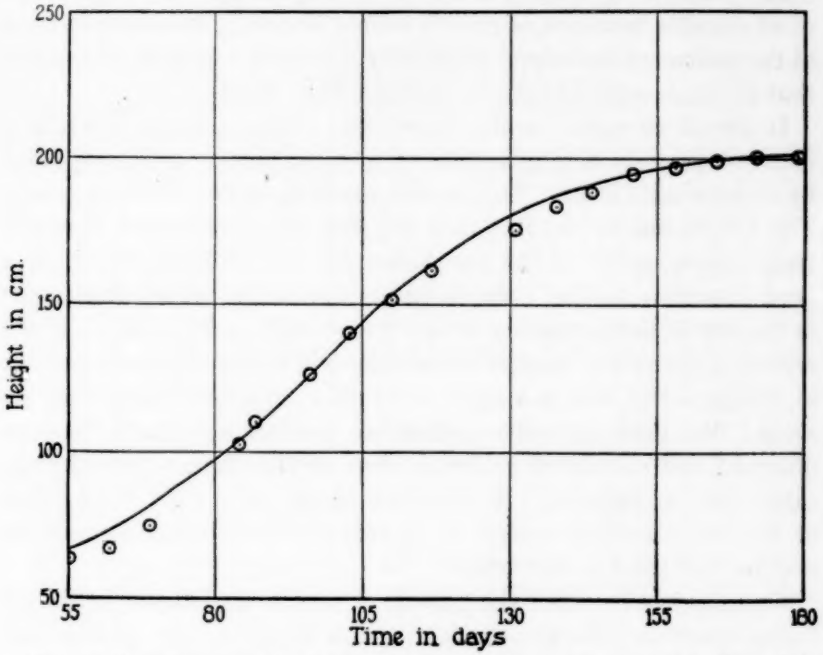

FIG. 4. Growth rate of *Juglans regia* trees. The curve represents the values obtained from the equation $\log \frac{x}{153.2-x_1} = K\,(t-96)$; points within circles represent the observed mean height at various intervals.

notes are concerned, is that *Juglans nigra* trees went into a condition of dormancy early in September, while *Juglans regia* trees, growing in adjoining rows and with similar care, grew until the middle of October.

This observation sustains the conclusion drawn from the foregoing studies on the rate of growth, and indicates that the extent of the growth cycle as well as the amount of growth is controlled by internal genetic factors.

The Reliability of Various Criteria for Determination of the Growth Rate.

It will be well to discuss certain questions which will arise in the mind of the reader. He desires to know, for example, whether the growth rate will conform to a differential equation when we use some other criterion, *e.g.* weight, as a measure of growth. An examination of all possible measures of growth should be made, especially in view of the statement sometimes heard that growth is a process so complex that no single equation can successfully represent it.

It should be noted at the outset that every measure which is a mean of separate determinations of mass or volume is accompanied by an inevitable error. This error is made up of two separate errors. The first is due to the fact that the individuals measured were not truly representative of the population, *i.e.* they did not constitute a good "random sample"; the second is due to the errors which arise in the use of the measuring device (meter stick or balances). If the errors are of a purely random nature they will largely offset one another in a large series, but in a small series they may materially affect the mean. We need not expect, therefore, to find agreement between observed and calculated values as close as the physical chemist obtains with his material. A difference of not more than 10 per cent of the calculated value may be regarded as sufficiently accurate to inspire confidence in the results.

Kreusler has recorded the growth of several varieties of maize at 7 day intervals. Height of plant, green weight (entire plant), and dry weight (entire plant) were determined on fairly large samples of plants. I have found that the growth rate of the different varieties determined by Kreusler follows the equation previously used

$$\frac{dx}{dt} = kx(a - x)$$

The determinations made upon one variety (Hühner-mais) will be given as an illustration. Those who are interested may profitably compute the growth rates of the other varieties which Kreusler followed. Reference to Table V shows undoubted errors in the weight of the samples at various dates. These can only be due to the use of too small a number of plants to give a true representation of the

population at that date. Table V shows a comparison of the growth rates of maize (Hühner-mais), with the height, green weight, and dry weight of the plants, recorded at intervals of 7 days. I have determined the value of K, the growth constant, for each measurement and, from the average value of all determinations, have calculated the value

TABLE V.

Rate of Growth of Maize.
Data taken from Kreusler (Hühner-mais).

t	Mean height.			Mean green weight.			Mean dry weight.		
	x (observed).	K	x (calculated).	x (observed).	K	x (calculated).	x (observed).	K	x (calculated).
days	*cm.*		*cm.*	*gm.*		*gm.*	*gm.*		*gm.*
7	8.0	0.0311	3.8						
14	12.7	0.0309	7.0				0.13	0.0536	0.09
21	17.4	0.0335	12.7	0.9	0.0665	1.1	0.19	0.0551	0.19
28	24.1	0.0374	22.2	2.5	0.0640	2.5	0.39	0.0547	0.43
35	36.4	0.0402	36.6	8.6	0.0605	7.2	1.10	0.0537	1.03
42	53.8	0.0472	55.5				2.97	0.0514	2.41
49	77.7	0.0453	76.2	53.4	0.0603	49.7	7.49	0.0486	5.59
56	99.0	0.0466	94.8	128.9	0.0454	111.5	15.37	0.0472	12.47
63	111.7	0.0441	108.7	193.4	0.0457	198.0	21.32	0.0688	25.03
70	124.0	0.0531	117.9	322.9	0.1119	278.0	43.13	0.0551	43.00
77	120.0	0.0339	123.2	304.2	0.0479	325.4	53.20	0.0301	60.97
84	127.7	0.0448	126.4	340.8	0.0703	346.7	79.63	0.0784	73.53
91	124.6	0.0297	128.1	353.8	0.0599	355.3	82.30	0.0641	80.41
Average		0.0398			0.0632			0.0551	

of x for corresponding values of t, using the tables prepared by Robertson (1910–15). The average divergence of the three sets of values is not large, particularly in view of the errors above mentioned. The results show a sufficiently good correspondence to inspire confidence in their reliability.

It will be seen that the growth rate of the maize plants follows the course of an autocatalytic reaction no matter whether we use as a criterion mean height, green weight, or dry weight. Their growth is similar to a chemical reaction in which one of the products of the reaction acts as a catalyst.

Attention may next be directed to some data recently published (Eckles and Swett) on the growth of heifers. The measurements

appear to have been carefully made and at regular intervals. They therefore afford good material for study. Eckles and Swett used various criteria for measuring the heifers. They concluded that height at the withers is the most reliable measure, though for comparative studies weight is also useful.

In computing the growth rate of these animals I have used a slightly different equation; *viz.*, $\frac{dx}{dt}=k(a-x)$ which upon integration becomes $x=a(1-e^{-kt})$.

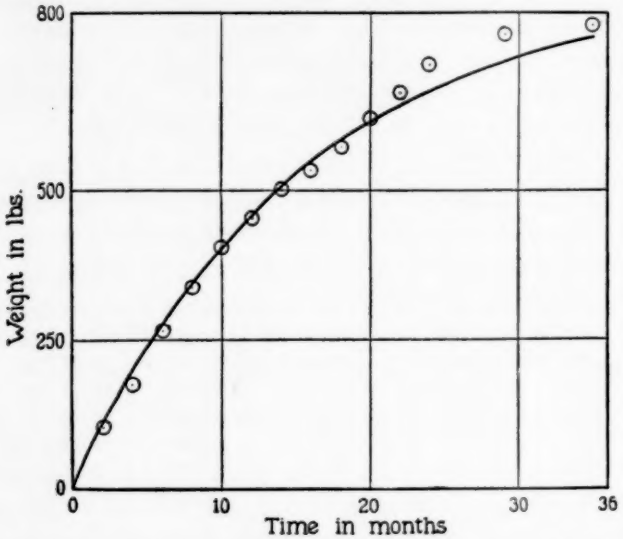

FIG. 5. Growth rate of Jersey heifers represented by mean weight. The curve shows the values obtained from the equation $x=850\ (1-e^{-0.065t})$; points within circles represent observed weights at intervals of 2 months.

The rate as thus expressed is directly proportional to the amount of growth yet to be made. It is therefore more rapid at the outset and slower near maturity than the formula used for computing the growth rate of maize in Table V. In the case of the heifers, a, the weight at 3 years of age, was found by a series of approximations to be 850 pounds, and k, the growth constant, was 0.065. The equation used for determining the calculated values of x was therefore $x=850(1-e^{-0.065t})$ (Fig. 5).

It will be seen that the observed and calculated values of x agree very well, especially when one bears in mind the extent of variability in biological material. In the latter part of the period the observed values are consistently higher than the calculated. These weight differences are plainly due to the increase in weight during pregnancy. The first parturition occurred at 30 months. As it is, however, the root-mean-square deviation between observed and calculated weights is only 20 pounds.

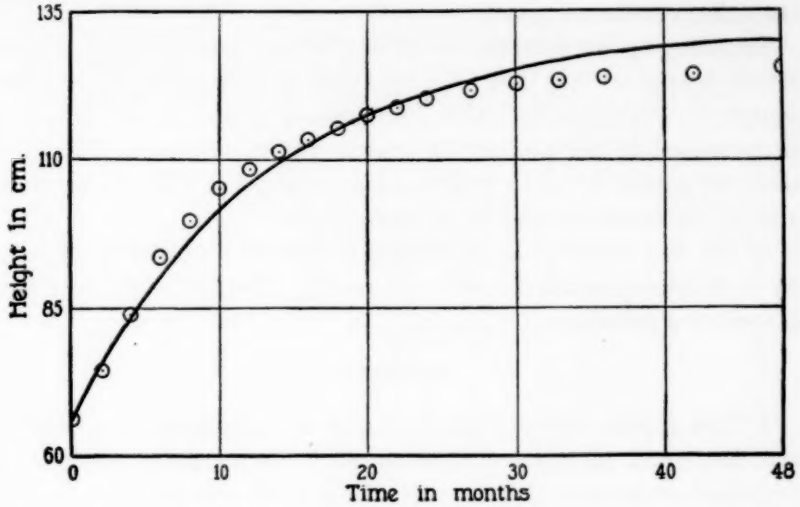

FIG. 6. Growth rate of Jersey heifers represented by height at withers. The curve shows the values obtained from the equation $x = 128\,(1 - e^{-0.08\,(t+9)})$; points within circles represent observed heights at various intervals.

Inquiry may next be directed to the rate at which these animals increased in size and to the value of different kinds of measurements. Eckles and Swett found that the most reliable size measurement was the height of the animal at the withers. The mean height at birth was 66.1 cm.; at 4 years it was 125.6 cm. It thus appears that at birth the calf is a little more than half as tall as the mature cow. When we come to study the growth rate it is evident that we cannot use the postnatal life period as the complete growth cycle, because the animal has attained over half its height before birth. Let us assume that height growth begins near the beginning of the gestation period, then

we shall use $t+9$ as the time instead of t, the observed time from birth. By a series of approximations, 128 was taken as the value of a, and 0.08 as the value of k. The formula used was then $x = 128\ (1-e^{-0.08\ (t+9)})$ (Fig. 6). The agreement for the most part is strikingly close. The close correspondence between the observed heights and those calculated by the formula bears out the observation of Eckles and Swett that the growth of the skeleton of the cow is less influenced by conditions of gestation and lactation than the other criteria of growth.

Summing up the data on the growth rate of Jersey heifers as given above, it appears that they grow by a definite rate which can be represented as a reaction in which the increase in any given interval is proportional to the amount of growth yet to be accomplished. It does not greatly matter whether one uses height at withers or weight, though the former is slightly more accurate.

In the case of maize and of heifers it appears that growth proceeds by a definite quantitative rate, no matter whether height or weight is used as a criterion.

SUMMARY.

1. The growth rate of organisms may be considered as a chemical reaction which gives the mature organism as its end-product. The organism grows at a definite rate which is, at any moment, proportional to the amount of growth yet to be made.

2. Shoots of young pear trees measured at weekly intervals during the growing season showed a rate similar to that of an autocatalytic reaction.

3. Young walnut trees showed distinct cycles of growth in a single season, but the growth in each cycle proceeded at a rate corresponding to an autocatalytic reaction.

4. The growth rate follows a definite, quantitative course though judged by different criteria. Data are presented for maize in which green weight, dry weight, and height of the plant are used. Data for cattle show that either weight or height of the animal may be used as a criterion.

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ON THE CAUSE OF THE INFLUENCE OF IONS ON THE RATE OF DIFFUSION OF WATER THROUGH COLLODION MEMBRANES. II.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, February 28, 1920.)

I.

In this note it is intended to complete the proof that the influence of the concentration of electrolytes on the transport of water through a collodion membrane is similar in the case of free and of electrical endosmose.

Fig. 1 gives the curves representing the influence of different concentrations of KCl, K_2SO_4, K_3 citrate, and $CaCl_2$ upon the initial rate of diffusion of water from pure water through a collodion membrane into solution (free osmosis). The solution was inside a collodion bag and the latter dipped into a beaker filled with distilled water. The ordinates of the curves give the rise in the level of liquid after 20 minutes in a glass tube pushed through a rubber stopper into the collodion flask. The curves show, as pointed out in a preceding publication,[1] that the level rises at first with increasing concentration until it reaches a maximum at about M/512 or M/256 and that it then drops with a further rise in concentration until the latter is M/16 when a second rise begins. The second rise is presumably the expression of a prevalence of the gas pressure effect while the first rise and fall are the effect of the electrostatic influence of the ions on the rate of diffusion of water. As stated in the preceding publication, pure water as well as water containing electrolytes is positively charged when in contact with a collodion membrane while the latter is negatively charged. Leaving aside the gas pressure effect on the rate of diffusion, water will be driven through the pores or interstices of the

[1] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 173.

membrane from that side of the membrane which has a smaller density of charge to that side which has a greater density. The density of charge on a collodion membrane in contact with a watery solution is influenced in an opposite sense by the oppositely charged ions of an electrolyte in solution, the density being increased by that ion which

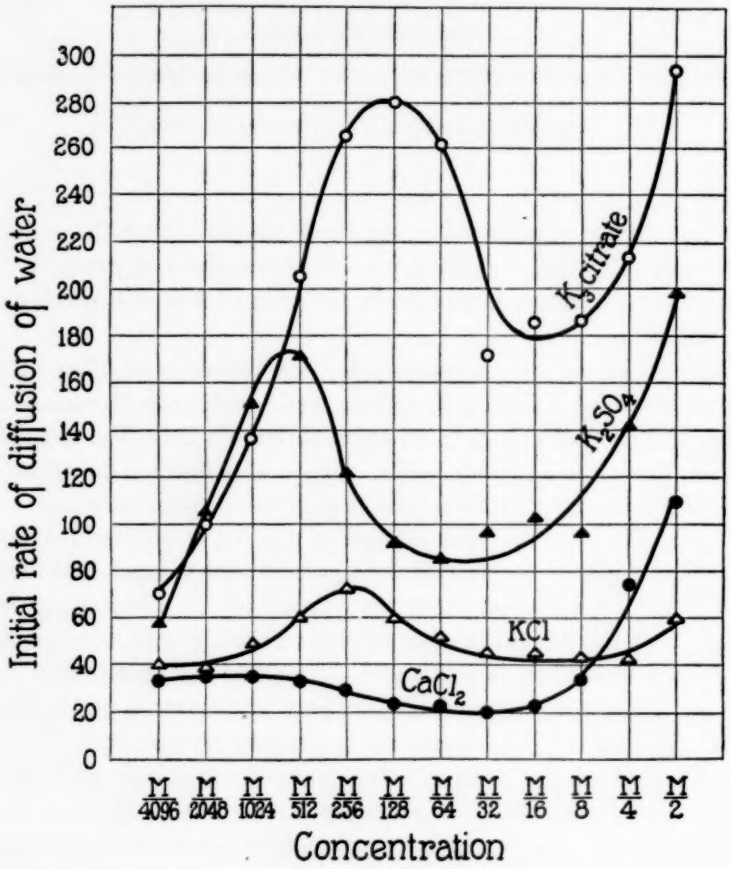

FIG. 1. *Free osmosis.* Effect of ions on free osmosis when water is positively charged. Inside the collodion bag, solutions of $CaCl_2$, KCl, K_2SO_4, and K_3 citrate in different concentration; outside, H_2O. Abscissæ are the logarithms of concentrations of electrolyte in the collodion bag, ordinates the rise in level of liquid in manometer after 20 minutes (with the exception of K_3 citrate which is given after 10 minutes).

has the same sign of charge as the membrane and being diminished by that ion which has the opposite sign of charge.[2] Since the collodion membrane is negatively charged in contact with the solutions mentioned in Fig. 1, the density of charge on the membrane is increased by the anion and diminished by the cation of the electrolyte.

The relative influence of the oppositely charged ions of an electrolyte is, however, *not* the same for different concentrations. When the membrane is negatively charged the influence of the anion on the charge of the membrane increases in lower concentrations more rapidly with increasing concentration than the depressing effect of the cation until the concentration is M/512 or M/256. When this point is reached, the depressing influence of the cation on the negative charge of the membrane increases more rapidly with increasing concentration than the influence of the anion.

This explains why the curves representing the initial rate of diffusion of water from pure water through a collodion membrane to a solution of an electrolyte rise at first with increasing concentration until the concentration is about M/512 or M/256 and then generally drop with a further increase in concentration.[3] The rise of the curves is higher the higher the valency of the anion and less the higher the valency of the cation. In the case of $CaCl_2$ the strong depressing effect of Ca prevents a rise by the Cl ion.

It follows from the theoretical discussion in the preceding paper[2] that if this explanation of the influence of the concentration of the electrolyte is correct, the rate of diffusion of water through a collodion membrane must vary in the same sense with the concentration of the electrolyte in the case of *electrical* endosmose as it does in the case of *free* osmosis. This is shown to be true by the curves in Fig. 2. These curves represent the relative transport of water in the case of electrical endosmose through a collodion membrane in the presence of the same electrolytes as those used in Fig. 1.

In these experiments the collodion flasks were filled with a solution of an electrolyte and were dipped into a beaker containing an identical solution. A large disc of platinum at the bottom of the beaker

[2] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 387.

[3] The second rise beginning with M/16 is due to the gas pressure effect and will not be discussed in this paper.

was one electrode while the other electrode was a platinum wire pushed through a glass tube into the collodion flask. The latter was closed with a rubber stopper through which the glass tube with a bore of 2 mm. diameter was pushed into the collodion flask. The distance between the two electrodes was 7.0 cm. A P.D. of 15 volts was applied and the current through the collodion membrane increased slowly in intensity until it finally became fairly constant. To accelerate this process a P.D. of 200 volts was used for 30 seconds or a little longer, and then the P.D. was changed to 15 volts. This, however, was done only in the case of the more dilute solutions. The

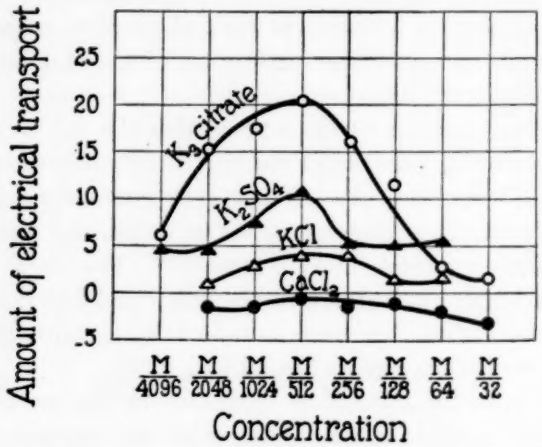

FIG. 2. *Electrical endosmose.* Effect of the same electrolytes as in Fig. 1 on electrical endosmose. Inside and outside the collodion bag identical solutions. Abscissæ are the logarithms of concentrations of electrolyte in solution, ordinates rise of level of liquid on the side of the cathode in 15 minutes. The influence of electrolytes on the rate of transport of water in electrical endosmose is similar to that in free osmosis (Fig. 1).

liquid rose in the glass tube when it contained the cathode, showing that the watery phase was positively charged. The rise in level in the glass tube was measured for 15 minutes (usually between 5 and 20 minutes after the commencement of the action of the current).

The reader will notice that the amount of liquid transported by the current rises at first with the increase in concentration of the solution until the latter is about M/512 and that with a further increase in

concentration the quantity of electro-endosmotic transport falls. The curves resemble those in Fig. 1 except that the maximum is a little lower in the case of electrical endosmose. The writer is inclined to explain this difference by the fact that in electrical endosmose the concentration of the liquid is increased by the secondary chemical reactions at the electrodes and that thereby in a M/512 solution of K_2SO_4 or KCl the concentration of the solution rises gradually as a consequence of electrolysis and secondary chemical reactions at the electrodes and approaches M/256.

It is, moreover, obvious that the electrical transport (Fig. 2) of the positively charged liquid increases with the valency of the anion as in free osmosis (Fig. 1) and that it diminishes with the increasing valency of the cation as shown by the flat curve for $CaCl_2$. The curves for $MgCl_2$ and $BaCl_2$ are like those for $CaCl_2$ in the case of free osmosis as well as in the case of electrical endosmose.

We have inferred in the preceding paper[3] that the rate of transport of liquid in electrical endosmose varies, if the other conditions remain equal, with the value of the charge ϵ on the unit area of membrane. If this inference is correct then it follows from the nature of the curves in Fig. 2 that beginning with the lowest concentrations the influence of the anion on the density of charge of the membrane increases at first more rapidly with increasing concentration than the depressing effect of the cation upon the density of this charge, while later the reverse occurs. The turning point seems to lie for the solutions mentioned between M/512 and M/256, where it also lies for free osmosis.

II.

When we separate solutions of electrolytes with a hydrogen ion concentration of 10^{-4} N or above from pure water by collodion membranes which have been treated with a protein, the watery phase of the double layer is negatively and the membrane positively charged.[1,4] In the preceding paper[3] it was shown that in such a case the charge of the membrane is increased by the cation and diminished by the anion of an electrolyte in solution, both effects increasing with the valency

[4] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 717.

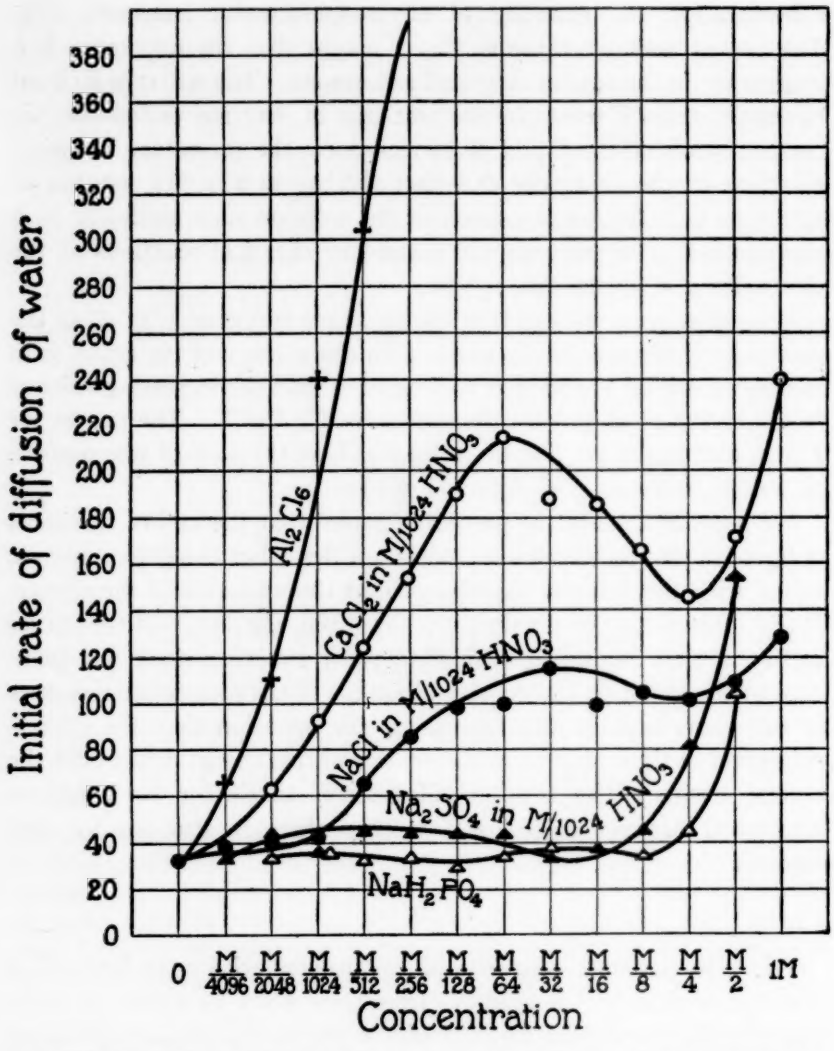

FIG. 3. *Free osmosis.* Effect of ions on free osmosis when water is negatively charged due to acid reaction of solution. Inside the collodion bag acidulated solutions of salts; outside distilled water. Abscissæ are the logarithms of concentration of solution, ordinates rise of level of liquid in manometer after 20 minutes.

of the ion. Fig. 3 represents the influence of different concentrations of electrolytes (with hydrogen ion concentration above 10^{-4} N) upon the rate of diffusion of water through the membrane. It increases with the valency of the cation and diminishes with the valency of the anion, the curves in the case of Na_2SO_4 or NaH_2PO_4 showing no rise. The curves for $CaCl_2$ and NaCl show a rise and then a drop, the turning point, however, lying at higher concentrations than for neutral solutions. The rise and drop find their explanation on the assumption that the influence of the cation on the charge of the membrane

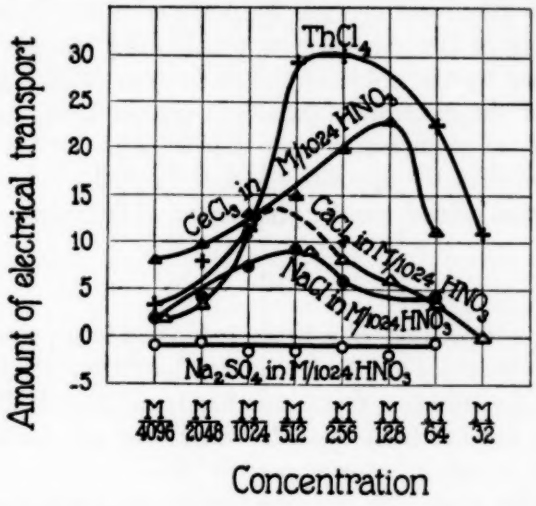

FIG. 4. *Electrical endosmose.* Effect of acidulated salt solutions on electrical endosmose. Inside and outside the bag indentical solutions. Similarity of curves of transport in Fig. 4 and Fig. 3.

increases at first more rapidly with increasing concentrations than the depressing effect of the anion upon this charge, while at concentrations above a certain point the reverse happens.

This conception is supported by experiments on electrical endosmose as represented in Fig. 4. The solutions of Na_2SO_4, NaCl, $CaCl_2$, and $CeCl_3$ were made up in M/1,024 HNO_3 and the hydrogen ion concentration was in the neighborhood of 10^{-3} N. The $ThCl_4$ solution was sufficiently acid on account of hydrolysis (its pH varying between 3.5 and 1.9 according to concentration). The watery phase was negatively charged and it was necessary to put the anode into the glass

tube in order to bring about a rise in the level of water in the tube. It is obvious from Fig. 4 that the electro-endosmotic transport of the negatively charged liquid rises at first with increasing concentration of the electrolyte and then falls again; and the initial rise increases with increasing valency of the cation and diminishes with increasing valency of the anion. The turning point varies for different electrolytes, probably on account of secondary chemical reactions at the electrodes, especially acid formation at the anode. The curves if interpreted on the basis of Helmholtz's theory prove that when the membrane is positively charged its positive charge is raised in low concentrations of electrolytes more considerably by the cation than it is depressed by the anion of the electrolyte, while when the concentration of the electrolyte exceeds a certain limit the depressing effect of the anion increases more rapidly with further increase in concentration than the opposite effect of the cation; thus supporting the explanation offered for the phenomena of free osmosis in Fig. 3.

III.

Fig. 5 is a repetition of a figure published in a preceding paper[5] showing the difference of influence of Al_2Cl_6 on the rate of diffusion of water when the collodion membrane has been treated with a protein and when it has not been treated. When the membrane has been treated with a protein, water is powerfully attracted by a solution of Al_2Cl_6 and the attraction increases with the concentration (upper curve); when the membrane has not been treated with a protein, water is not attracted by the solution except when the concentration becomes so high that the gas pressure effect of the solution shows itself (lower curve). The explanation offered by the writer was as follows. When we separate Al_2Cl_6 solutions of sufficiently low concentration from pure water by a membrane treated with gelatin, the water diffuses through the membrane in the form of negatively charged particles which are attracted powerfully by the Al ion and repelled weakly by the Cl ion; or, in other words, the Al ion increases the positive charge of the membrane considerably and the Cl ion depresses the same charge less. As a consequence the density of the positive charge of the membrane on the solution side of the membrane must

[5] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 255.

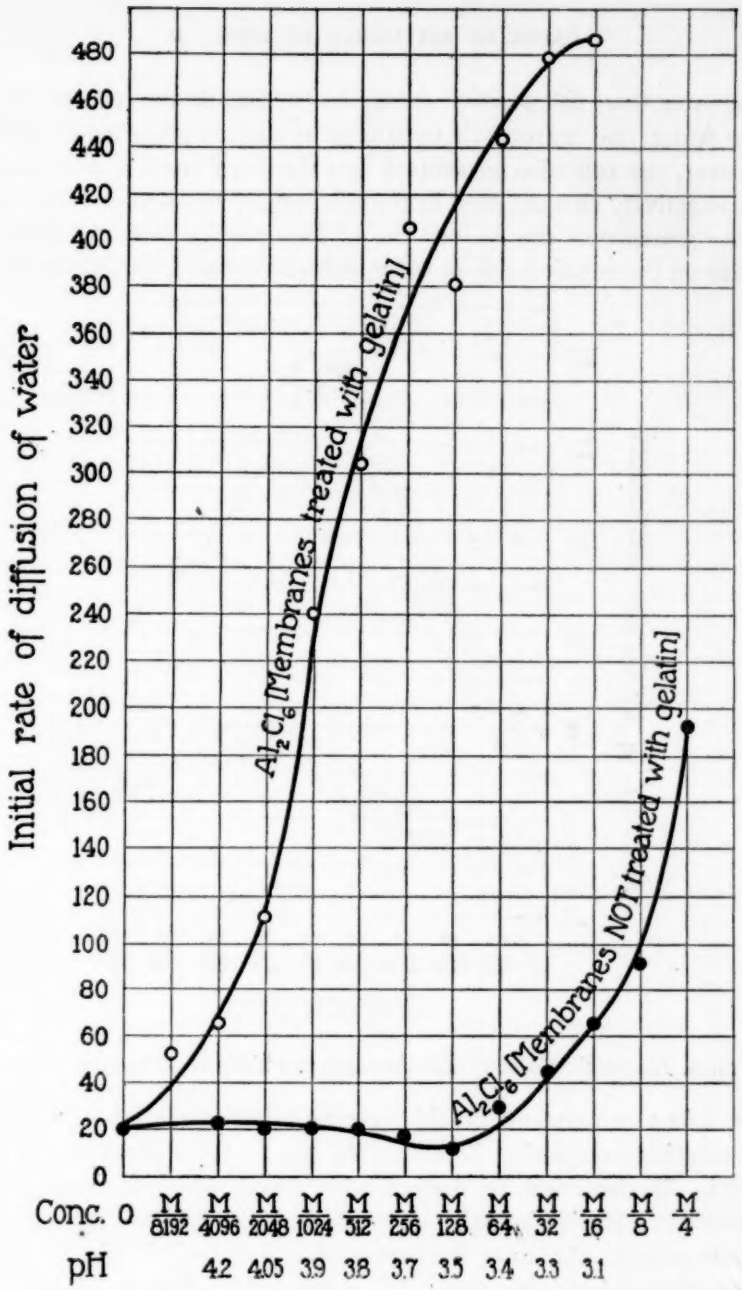

FIG. 5. *Free osmosis.* Upper curve, influence of Al_2Cl_6 upon the rate of diffusion of water when the collodion membrane has been treated with gelatin; lower curve, influence of Al_2Cl_6 on rate of diffusion when the membrane has not been treated with gelatin.

be greater than the positive charge of the membrane on the side of pure water and water will be driven to the solution side. When, however, the collodion membrane has not been treated with gelatin it is negatively charged even in the presence of Al_2Cl_6 and of acid, and in the presence of the Al ions the Cl ions cannot raise the negative charge on the solution side of the membrane beyond that on the water

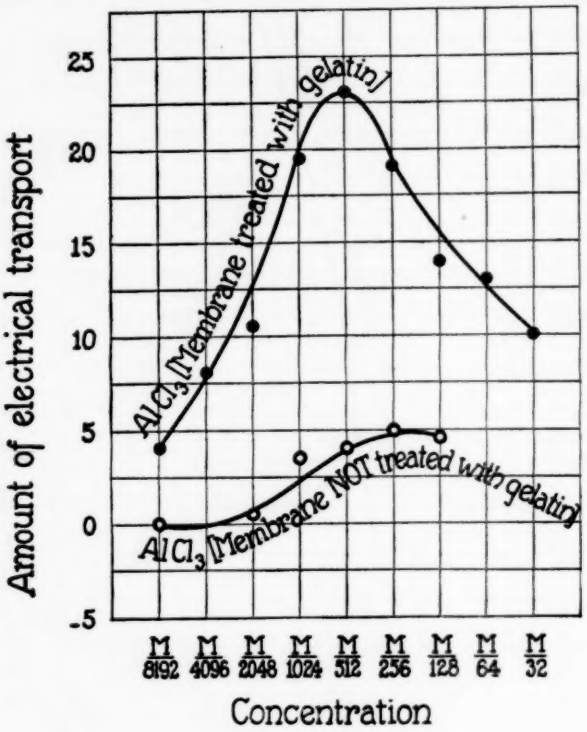

FIG. 6. *Electrical endosmose* with the same two kinds of membrane.

side. As a consequence in this case no other attraction of water by the solution except that based on the gas pressure effect can be produced. If this view is correct, the curves of electro-endosmotic transport of liquid through membranes treated and not treated with gelatin must be similar to the curves of free osmosis in Fig. 5. Fig. 6 shows that this is the case. When identical solutions of $AlCl_3$ are put into the collodion flask and into the beaker into which the collodion flask is dipped, and when a P.D. is applied in the way described,

a transport of water to the anode occurs which increases with increasing concentration to about M/512 provided the membrane had been treated with a protein (upper curve, Fig. 6); when the collodion membrane has not been treated with a protein no such migration occurs (lower curve, Fig. 6). Hence the curves for electro-endosmotic transport of liquid through collodion membranes and the transport of water in free osmosis run parallel, supporting the explanation offered.

In comparing the upper curves in Figs. 5 and 6, the reader will notice a difference, inasmuch as in the case of free osmosis through membranes treated with gelatin (upper curve, Fig. 5) the curve shows only a rise but no drop, while in the upper curve for electro-endosmotic transport (Fig. 6) through a gelatin-treated membrane in the presence of a solution of $AlCl_3$ there occurs the characteristic drop at a concentration beyond M/512. The writer is inclined to attribute the reason for this difference to the fact that in free osmosis the gas pressure effect prevents the drop while in electrical endosmose this gas pressure effect is excluded (since the solutions on both sides of the membrane are identical).

IV.

When we separate a M/256 solution of Na_2SO_4 or Li_2SO_4 by a collodion membrane from pure water, the latter will diffuse into the solution with a certain velocity. When we add small and identical quantities of a salt like KCl to the solution of M/256 Na_2SO_4 and to the distilled water, this velocity will be diminished;[6] owing to the fact that beyond a concentration of M/256 Na_2SO_4 the repelling or depressing effect of the cation of the solute increases more rapidly with increasing concentration of the electrolyte than the attracting or accelerating effect of the anion on the rate of diffusion. When we add $MgCl_2$ or $CaCl_2$ instead of KCl the depressing effect is still greater than in the case of KCl, owing to the fact that Mg and Ca, as bivalent ions, have a greater depressing effect than K. Fig. 7 illustrates this statement, showing that the addition of KCl or $MgCl_2$ or $CaCl_2$ to M/256 Na_2SO_4 diminishes the rate of diffusion of liquid into the Na_2SO_4 solution and that $MgCl_2$ and $CaCl_2$ act more power-

[6] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 273.

fully than KCl. The addition of 0.2 cc. of M/4 $MgCl_2$ or $CaCl_2$ to 100 cc. of M/256 Na_2SO_4 (concentration of $MgCl_2$ or $CaCl_2$ = M/2,000) lowers the rate of diffusion of water into the solution more than 50 per cent, while the effect of the addition of 0.2 cc. of M/4 KCl (concentration of KCl = M/2,000) lowers the rate of diffusion of water into the solution considerably less.

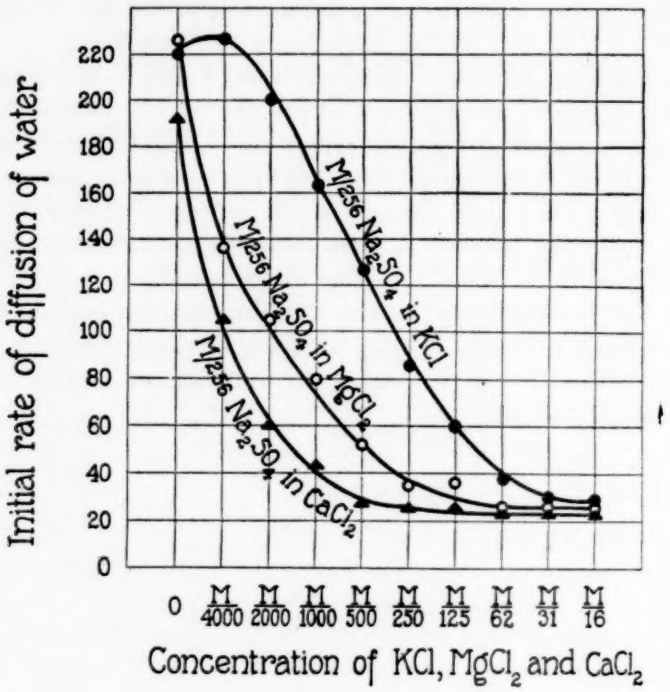

FIG. 7. *Free osmosis.* Depressing effect of the addition of KCl, $MgCl_2$, and $CaCl_2$ upon the attraction of water by M/256 Na_2SO_4.

Fig. 8 shows that the addition of KCl and $CaCl_2$ to M/512 Na_2SO_4 has the same depressing effect on the rate of transport of liquid through the collodion membrane in the case of electrical endosmose. In this, as in the preceding experiments on electrical endosmose, identical solutions were put into both sides of the collodion membrane. These solutions were M/512 Na_2SO_4 alone or with the addition of small quantities of KCl or $CaCl_2$. A P.D. of 15 volts was produced on the opposite sides of the membrane and the rate of transport of water

(which took place to the cathode) was observed. The curves in Fig. 8 show the result. The quantity of transport of liquid was depressed both by the addition of KCl and of $CaCl_2$ but more by $CaCl_2$ than by KCl. The addition of 0.1 cc. of M/4 $CaCl_2$ to 100 cc. of M/512 Na_2SO_4 (concentration of $CaCl_2$ = M/4,000) lowers the transport of water more than twice as much as the addition of 0.1 cc. of M/4 KCl. The depressing influence of the addition of KCl or $CaCl_2$ in the electrical transport of water through a collodion membrane can be demonstrated equally well with M/256 solutions of Na_2SO_4 as with M/512 solutions.

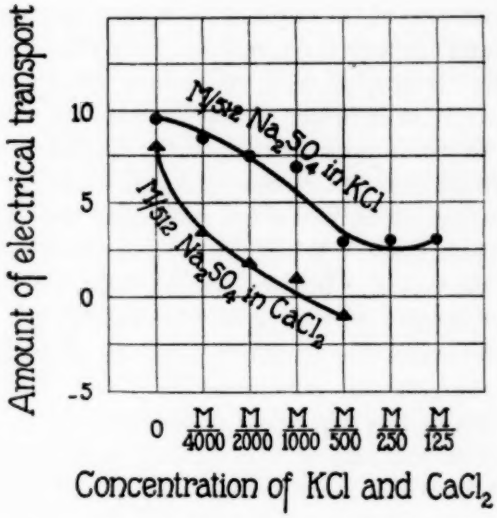

FIG. 8. *Electrical endosmose.* Showing that a similar depressing effect as in Fig. 7 exists in the case of electrical endosmose.

These experiments on electrical endosmose show that the density of negative charge on the membrane in contact with a M/256 or M/512 Na_2SO_4 solution is diminished by the addition of any electrolyte (whose anion does not act more powerfully than SO_4) and that the diminution increases with increase in the valency of the cation added. This furnishes the explanation of the fact that when M/256 Na_2SO_4 is separated from pure water by a collodion membrane the rate of diffusion of the positively charged water into the solution is diminished by the addition of KCl and still more by the addition of $MgCl_2$ or $CaCl_2$.

The writer has duplicated the majority of the experiments he has thus far published on free osmosis by experiments on electrical endosmose and there is a fair degree of similarity in the two cases.

SUMMARY.

1. It had been shown in previous publications that when pure water is separated from a solution of an electrolyte by a collodion membrane the ion with the same sign of charge as the membrane increases and the ion with the opposite sign of charge as the membrane diminishes the rate of diffusion of water into the solution; but that the relative influence of the oppositely charged ions upon the rate of diffusion of water through the membrane is not the same for different concentrations. Beginning with the lowest concentrations of electrolytes the attractive influence of that ion which has the same sign of charge as the collodion membrane upon the oppositely charged water increases more rapidly with increasing concentration of the electrolyte than the repelling effect of the ion possessing the opposite sign of charge as the membrane. When the concentration exceeds a certain critical value the repelling influence of the latter ion upon the water increases more rapidly with a further increase in the concentration of the electrolyte than the attractive influence of the ion having the same sign of charge as the membrane.

2. It is shown in this paper that the influence of the concentration of electrolytes on the rate of transport of water through collodion membranes in electrical endosmose is similar to that in the case of free osmosis.

3. On the basis of the Helmholtz theory of electrical double layers this seems to indicate that the influence of an electrolyte on the rate of diffusion of water through a collodion membrane in the case of free osmosis is due to the fact that the ion possessing the same sign of charge as the membrane increases the density of charge of the latter while the ion with the opposite sign diminishes the density of charge of the membrane. The relative influence of the oppositely charged ions on the density of charge of the membrane is not the same in all concentrations. The influence of the ion with the same sign of charge increases in the lowest concentrations more rapidly with increasing concentration than the influence of the ion with the opposite sign of charge, while for somewhat higher concentrations the reverse is true.

THE REVERSAL OF THE SIGN OF THE CHARGE OF MEMBRANES BY HYDROGEN IONS.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, March 13, 1920.)

I. INTRODUCTION.

The similarity between the influence of ions on free and on electrical endosmose through collodion membranes leads to the conclusion that this influence is due to an alteration of the density of the electrical charge of the membrane by the ions.[1] This conclusion rests on the assumption that the Helmholtzian theory of the double layer and the formula connecting the volume of transport of liquid with the density of charge is correct. We have seen that on this assumption the observations lead to the conclusion that the ions with the same sign of charge as the membrane increase and the ions with the opposite sign of charge diminish the density of charge of the membrane. Whether, therefore, a given ion increases or diminishes the density of charge of the membrane depends upon the sign of the charge of the membrane, and, hence, the sign of the charge precedes the influence of ions on the density of charge. One of the problems in the theory of the double layer at the boundary of membrane and watery solution is therefore the origin of the sign of the charge of membranes in contact with water.

Chemists consider the double layer at the boundary of membrane and water as ionic in character and as due to the preferential adsorption of one kind of ions by the membrane, while the watery phase of the double layer is formed by the ions with the opposite charge from those adsorbed. This view has been developed with remarkable lucidity and consistency by Perrin[2] who assumes that the H and OH

[1] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 387, 563.

[2] Perrin, J., *J. chim. physique*, 1904, ii, 601; 1905, iii, 50; Notice sur les titres et travaux scientifiques de M. Jean Perrin, Paris, 1918.

ions are especially influential in determining the sign of charge of the membrane. In alkaline solutions the OH ions are said to be adsorbed by the membrane, while the mobile, watery stratum of the double layer is formed by the cations; in acid solutions the H ions are thought to be adsorbed while the anions form the mobile stratum of the double layer. When the OH ions are adsorbed by the membrane the latter is negatively charged, when the H ions are adsorbed the membrane is positively charged. This view meets a difficulty in the fact that as a general rule membranes are negatively charged when in contact with neutral water. The adsorption hypothesis meets this difficulty with an additional assumption; namely, that in a neutral solution the OH ions have a greater tendency to be adsorbed by a membrane than the H ions. We should, however, be forced to assume that the preferential adsorption of OH ions occurs also in some cases in acid solutions, since we have seen that collodion membranes (not treated with a protein) are negatively charged even in strong acid solutions.[3] Another difficulty was pointed out by Perrin himself, namely that no other monovalent ion except the H and OH ions were able to reverse the sign of charge of a membrane, and he intimated that this might be due to the fact that the velocities of H and OH ions are greater than those of any other ion. But if the velocity determines the relative degree of adsorption of ions then the H ions should be more readily adsorbed by a membrane in neutral solutions than the OH ions.

A second view, which is held chiefly by physicists, considers the formation of an electrical double layer at the boundary of membrane and water as a case of contact electricity, which may be influenced but which need not be caused by ions. Lenard[4] has shown that when very minute particles are torn off from the free surface of water the minute particles are negatively charged while the water assumes a positive charge. He concludes from this that at the surface of the water there exists an electrical double layer the external stratum of which is negatively charged, while the internal stratum is positively charged. Since he was able to show that such a double layer exists at the surface of water even in a vacuum, this double layer cannot

[3] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 255.
[4] Lenard, P., *Ann. Physik*, 1915, xlvii, 463.

have its origin in an adsorption of ions. He assumes that the double layer at the boundary of water and membrane is also entirely inside the watery phase. This seems, however, to be disproved by an experiment of Coehn and Franken.[5] A sphere of paraffin dipped into water possesses a high negative charge which can be demonstrated electrometrically when the paraffin is lifted out of the water. If, however, a film of water is caused to adhere temporarily to the surface of the paraffin when it is lifted out of the water, the electrometer indicates no charge as long as this film exists, but indicates a charge the moment the film has disappeared. This leaves no doubt that when a solid is bounded by water the one charge is in the membrane and the opposite charge in the watery phase. The fact that paraffin and solid substances in general are negatively charged when in contact with water is ascribed by Coehn to the difference in the dielectric constant of the two phases. Coehn has found that substances of a higher dielectric constant assume a positive charge when in contact with a substance of a lower dielectric constant. This would explain why membranes in general assume a negative charge when in contact with water since the dielectric constant of the latter is relatively high.

The formation of a double layer at the boundary of two phases is thus, according to Coehn, a phenomenon of contact (or frictional) electricity. Lenard and more recently Frenkel[6] have offered suggestions concerning the origin of the double layer which make it dependent on the Rutherford model of the atom. Lenard points out that the atoms at the surface of a body are generally oriented in such a way that the electrons are at the surface and the more massive part (the positive nucleus) is towards the interior. This idea has been elaborated by Frenkel[6] into a theory of surface electric double layers of solid and liquid bodies. According to this theory "double layers must exist on the surface of all liquid and amorphous solid bodies, whatever their chemical constitution. The latter will determine but the magnitude and distribution of electric charges on both sides of the surface."

[5] Coehn, A., and Franken, J., *Ann. Physik*, 1915–16, xlviii, 1005.
[6] Frenkel, J., *Phil. Mag.*, 1917, xxxiii, 297.

On the basis of this theory it seems natural that membranes should as a rule assume a negative charge when in contact with water. The fact which requires a further explanation is the possibility of a reversal of this sign of charge.

Perrin has shown that in certain cases acids are able to cause a membrane in contact with water to be positively charged. The fact that addition of alkali to an acid solution restores the original negative charge to such a membrane was explained by Perrin on the assumption that in an alkaline solution the OH ions are adsorbed. This latter assumption seems unnecessary since the addition of alkali to an acid may merely serve to lower the hydrogen ion concentration below the level required to make the membrane positive. It is, therefore, only necessary to explain why hydrogen ions in sufficient concentration impress a positive charge on so many membranes and the following is a contribution to the solution of this problem.

We have seen in the preceding papers of this series that a collodion membrane not treated with a protein always assumes a negative charge when in contact with a watery solution, even if this solution is acid. This was demonstrated in two ways, first by electrical endosmose,[1] and, second, by common osmosis.[3] The experiments with electrical endosmose do not lend themselves so well to the exact determination of that hydrogen ion concentration at which the membrane becomes positive on account of electrolysis and the secondary chemical reactions at the electrodes. The method of common osmosis is free from this source of error. When we separate a watery solution from pure water by a collodion membrane the pure water diffuses into the solution at a rate which is not only a function of the gas pressure of the solute but also of the electrostatic forces of the ions in solution. The rate increases with increasing valency of the anion and diminishes with increasing valency of the cation. When, however, the collodion membrane has been treated for some time with a protein it assumes a positive charge when the hydrogen ion concentration exceeds a certain value; and in this case the watery phase of the double layer assumes a negative charge. This is proved by the fact that when we separate a watery solution with a sufficiently high hydrogen ion concentration from pure water by a collodion membrane

treated with a protein, the water will diffuse into the solution at a rate increasing with increasing valency of the cation and diminishing with increasing valency of the anion. It is not difficult to find out by this method the critical hydrogen ion concentration at which the reversal in the sign of charge of the membrane occurs. The experiments to be described in this paper have led to the result that this critical hydrogen ion concentration is connected but not identical with the isoelectric point of the protein with which the collodion membrane has been treated.

Proteins are amphoteric electrolytes which can form salts with metals (metal proteinates, *e.g.* Na proteinate) as well as with the anions of acids (protein-acid salts, *e.g.* protein chloride). Whether they do the one or the other depends upon the hydrogen ion concentration of the solution. Below a certain hydrogen ion concentration the proteins form salts of the type of metal proteinates, *e.g.* Na gelatinate; above this critical hydrogen ion concentration they form salts of the type of protein-acid salts; *e.g.*, gelatin chloride.[7] Between the two concentrations proteins form salts with neither cation nor anion, and this is the so called isoelectric point. For a number of proteins (gelatin included) the isoelectric point lies in the neighborhood of a hydrogen ion concentration of 2×10^{-5} N; for oxyhemoglobin it lies at a very different hydrogen ion concentration; namely, 1.8×10^{-7} N. We will show in this paper that the hydrogen ion concentration required to produce a positive charge in a collodion membrane previously treated with a protein varies in the same sense as the isoelectric point of the protein used.

II. Membranes Treated with Gelatin.

The isoelectric point of gelatin is, according to Michaelis, at a hydrogen ion concentration of about 2×10^{-5} N (or in Sörensen's logarithmic symbol, pH = 4.7). A 1 per cent solution of gelatin was put into a collodion bag over night and was removed the next morning. The bag was rinsed a number of times (ten or twenty times or more) with water to remove all the gelatin except that film which remained apparently attached to the inner side of the col-

[7] Loeb, J., *J Gen. Physiol.*, 1918–19, i, 39, 237, 363, 483, 559.

lodion membrane. When such membranes were used for osmotic experiments it could be shown that the membrane had a positive charge as soon as the hydrogen ion concentration exceeded 10^{-4} N or a value slightly below 10^{-4}. The point of reversal was therefore near the isoelectric point of gelatin but on the acid side of that point.

The reader will remember that it had been shown in the preceding papers that when we separate a solution of an electrolyte by a collodion membrane from distilled water the water will be attracted by that ion which has the opposite sign of charge and will be repelled by the ion which has the same sign of charge as the watery phase of the double layer, and that both the attractive and repulsive effects increase generally with the valency of the ion.[1] In the case of a salt like $CaCl_2$ the repulsive effect of Ca upon positively charged water prevails over the attractive effect of Cl upon such water and no diffusion of such water into the $CaCl_2$ solution will occur; while when the water is negatively charged the attractive effect of Ca prevails over the repulsive effect of the Cl ion upon the water and water will diffuse rapidly into the solution. We therefore can use a solution of $CaCl_2$ to find out at which hydrogen ion concentration the reversal of sign in the charge of the watery phase occurs. When we separate a M/256 solution of $CaCl_2$ from a solution of water by a collodion membrane, water will commence to diffuse into the solution as soon as the water in contact with the membrane assumes a negative charge; while otherwise practically no such diffusion will occur. In these experiments the collodion flasks described in the previous experiments were used. They were closed with rubber stoppers perforated by a glass tube with a bore of 2 mm. in diameter serving as a manometer. The collodion bags were dipped into beakers filled with water. The M/256 solution of $CaCl_2$ and the water in the beaker into which the bags were dipped were always given the same hydrogen ion concentration; they were rendered acid by the addition of HNO_3 and alkaline by the addition of KOH. We can in this way ascertain at which hydrogen ion concentration the water will commence to diffuse into the solution of M/256 $CaCl_2$ and this will give us that hydrogen ion concentration where the reversal of the sign of charge on the membrane occurs. Such a curve is given in Fig. 1. The abscissæ are the hydrogen ion concentrations (expressed in terms of pH), the ordinates the rise of

level of liquid in the glass tube after 20 minutes. The reader will notice that the curve for M/256 $CaCl_2$ is low and flat as long as the pH > 4.0 and that a sharp rise in the curve begins at a pH of about 4.0 or a little less. The curve rises steeply with diminishing pH (*i.e.* increasing hydrogen ion concentration) until it reaches a maximum at

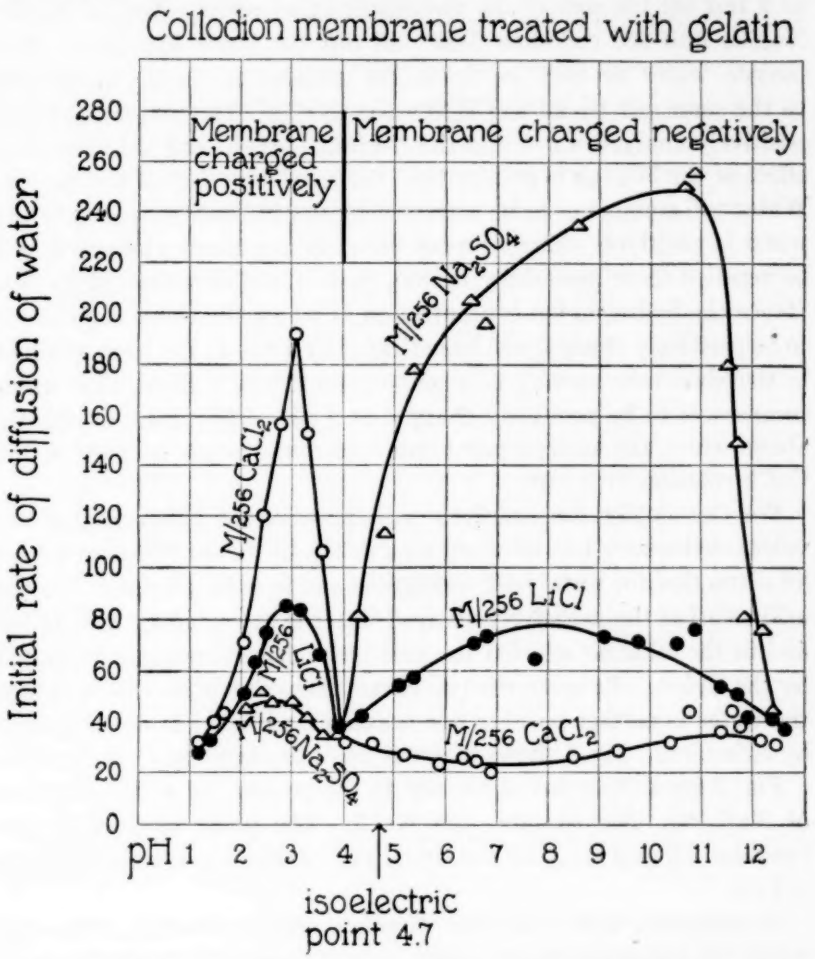

FIG. 1. *Collodion membranes previously treated with 1 per cent gelatin solution.* Abscissæ, pH (logarithms of hydrogen ion concentration with minus sign omitted). Ordinates, initial rate of diffusion of water from pure water through collodion membranes into solutions of salt indicated. Reversal of sign of charge of membrane at pH = 4.0; *i.e.*, on the acid side of the isoelectric point of gelatin.

about pH = 3.1 and then drops again. This drop is the concentration effect discussed in preceding papers and due to the ions with the opposite sign of charge as that of the membrane, in this case Cl and NO_3 (the latter being added with the nitric acid).[1]

Instead of using $CaCl_2$ we can also use a M/256 solution of Na_2SO_4 as a test for the sign of the electrification of water. We put M/256 Na_2SO_4 into the collodion flask and dip the latter into H_2O. The outside water as well as the M/256 solution of Na_2SO_4 is brought to the same pH by adding HNO_3 (or KOH). The SO_4 ion attracts positively charged water and the Na ion repels it, but the attractive effect of the SO_4 ion is greater than the repelling effect of the Na ion. Water will commence to be attracted by M/256 Na_2SO_4 as soon as the water is positively charged; when water is negatively charged it will be repelled more powerfully by SO_4 than it will be attracted by Na. Hence the hydrogen ion concentration at which the water commences to be positively charged will be indicated by a rise in the level of liquid in the glass tube serving as a manometer. Fig. 1 shows that water commences to be positively charged at a pH of about 4.0 or slightly above where the turning point was also found when we used M/256 $CaCl_2$ solution as a test.

We can finally use solutions with monovalent anion and monovalent cation as a test solution; *e.g.*, M/256 LiCl. In this case we get an attraction for water both in alkaline and in acid solutions. In the acid solution the negatively charged water is attracted by the Li ion and in the alkaline solution the positively charged water is attracted by the Cl ion. Between the two effects there should be a point where the water is neither positive nor negative and hence is not attracted by either ion. Fig. 1 shows that this point lies again at a pH near 4.0.

Fig. 2 gives the point of reversal in the presence of M/256 solutions of NaH_2PO_4, Na_2 oxalate, and NaCl. The point of reversal lies between 4.4 and 4.0, but the steep rise of the curve commences at pH 4.0.

Experiments with a number of other salts and other acids were made, all giving the same result; namely, that the membrane commences to be distinctly positively charged as soon as the pH is 4.0 or slightly above, but below 4.7. At the isoelectric point, pH = 4.7, no acid combines with the gelatin and no change of the sign of charge

can be expected at this point. When the hydrogen ion concentration of the solution becomes higher than this, an increasing amount of gelatin is transformed into gelatin-acid salt. With an increase in the concentration of the gelatin-acid salt an increasing proportion of the surface of the membrane assumes a positive charge. Hence the steep

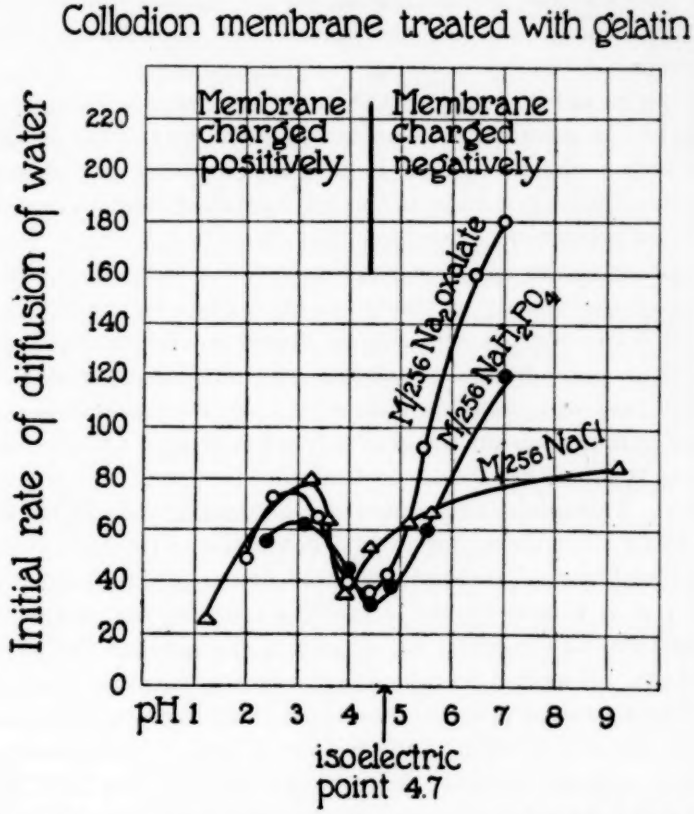

FIG. 2. *Collodion membranes treated with gelatin.* Reversal of sign of charge of membrane on acid side of the isoelectric point of gelatin.

rise of the curves in which $CaCl_2$ or $MgCl_2$ are used as test solutions must lie near the isoelectric point but on the acid side from the latter.

It may be pointed out that the influence of acids on the reversal of the sign of charge is not an additive effect of the oppositely charged

ions, as was the case in regard to the influence of ions on the density of charge of the membrane.[1] If this were the case, SO_4 should shift the steep rise of the curve more to the acid side and Ca more in the opposite direction. The curves given in this paper (and many other curves not published) show that this is not the case. This supports the view that the mechanism for the influence of ions on the density of charge and for the influence of the hydrogen ions on the reversal of the sign of charge is not the same.

The curves in Fig. 1 show that there are three relative minima of the charge of the membrane. One lies at about pH = 4.0 or slightly above, when the membrane is neither positively nor negatively charged. The drop leading to this minimum is not an additive function of the oppositely charged ions and differs in this respect from the drop leading to the other two minima, the latter being due to the diminution of the density of charge on the membrane caused by that ion which has the opposite sign of charge as the membrane. We have shown in the preceding experiments[1] that this depression occurs when the concentration of the electrolyte exceeds a certain value. One of these minima lies on the acid side, namely at a pH less than 2, owing to the fact that the concentration of the HNO_3 added is M/100 or more. This minimum is due to the depressing effect of the anions (NO_3 and SO_4) of the solution upon the density of the positive charge of the membrane. The third minimum lies at a pH of about 12 or above and is caused by the depressing effect of the cation of the solution, Na, K, etc., upon the density of the negative charge of the membrane. If we had continued to increase the concentration of acid there would have been another rise of the curve due to the gas pressure effect of the acid solution; and the same might have happened if we had been able to increase the concentrations of the KOH added; strong alkali solutions, however, dissolve the membrane.

III. Membranes Treated with Casein and Egg Albumin.

The isoelectric point of casein is, according to Michaelis,[8] identical with that for gelatin, since it lies at a pH of about 4.7. When we treat collodion membranes over night with a 1 per cent casein solution in-

[8] Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914.

stead of with gelatin solution, and make the same experiments as described for gelatin, we should expect to find the hydrogen ion concentration at which the collodion membrane treated with casein is rendered positive to be identical with that found for membranes treated

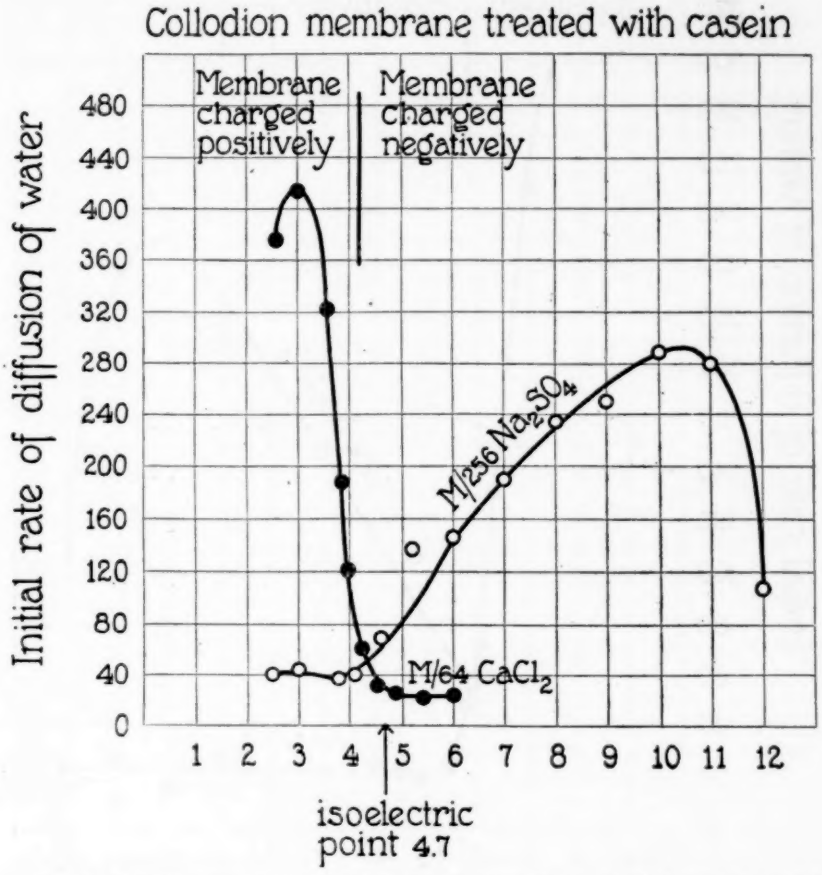

FIG. 3. *Collodion membranes treated with casein.* Reversal of sign of charge of membrane at pH below 4.7, on the acid side of the isoelectric point of casein.

with gelatin. Fig. 3 shows that this is correct. The curves for membranes treated with casein show a steep rise at a pH of 4.0 or slightly above, but below 4.7. The same is true for membranes treated with egg albumin (Fig. 4). The curves show a steep rise

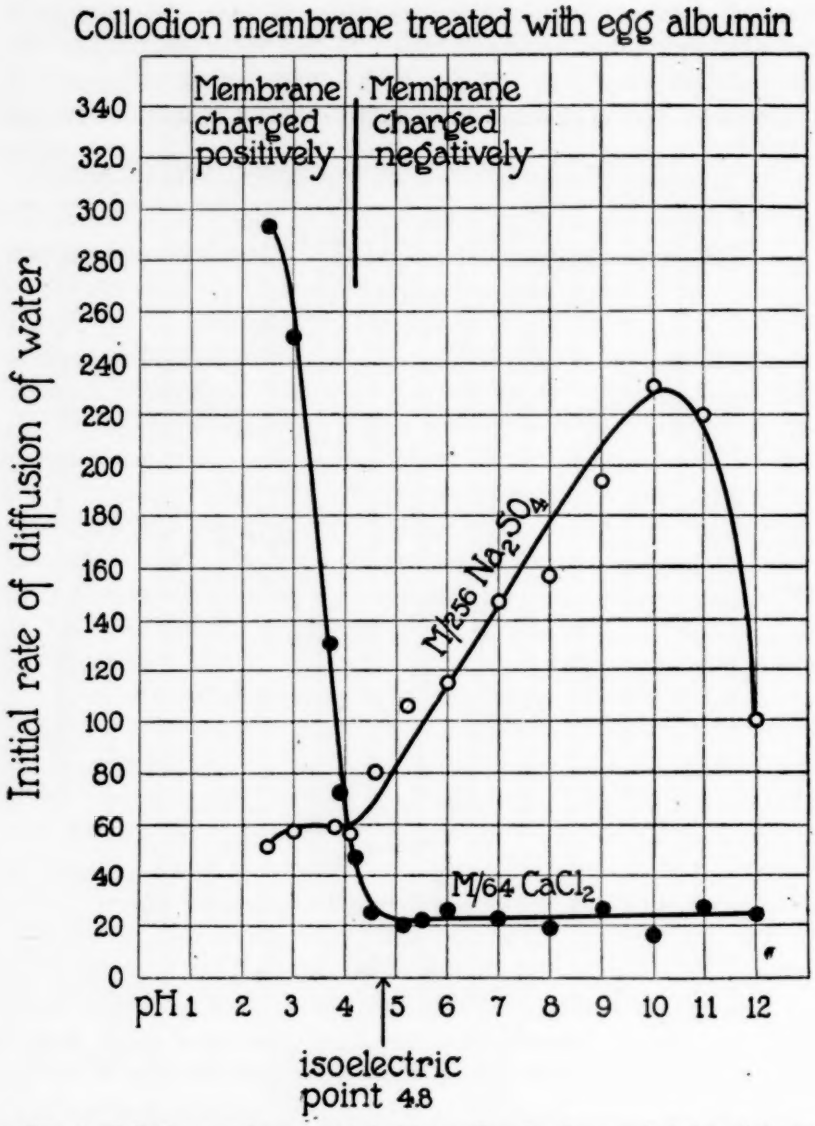

FIG. 4. *Collodion membranes treated with egg albumin.* Reversal of sign of charge of membrane at pH near 4.0, on the acid side of the isoelectric point of egg albumin.

at a pH of about 4.0. The isoelectric point of egg albumin is, according to Sörensen,[9] at 1.5×10^{-5} N or pH about 4.8.

IV. Membranes Treated with Oxyhemoglobin.

Defibrinated ox blood was diluted repeatedly with isotonic solutions of NaCl and centrifuged to remove practically all the proteins

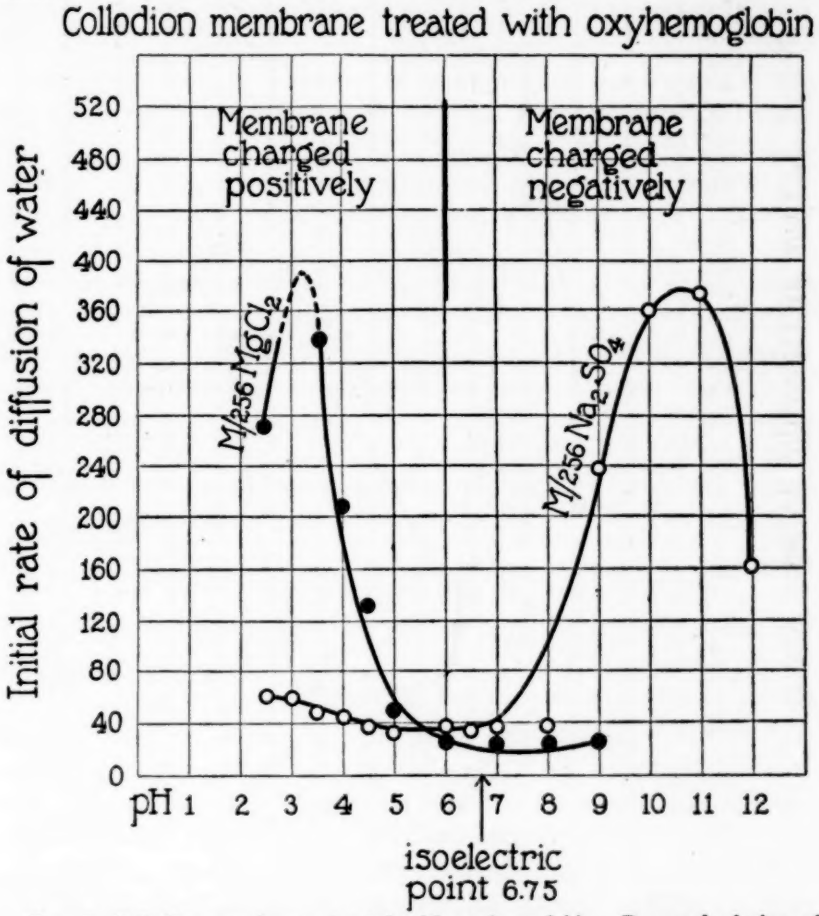

FIG. 5. *Collodion membranes treated with oxyhemoglobin.* Reversal of sign of charge of membrane at pH of about 6.0, on the acid side of the isoelectric point of oxyhemoglobin.

[9] Sörensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1915–17, xii.

of the blood except those contained in the red corpuscles. After repeating this process five or more times the corpuscles were laked by adding four times their volume of distilled water. Newly prepared

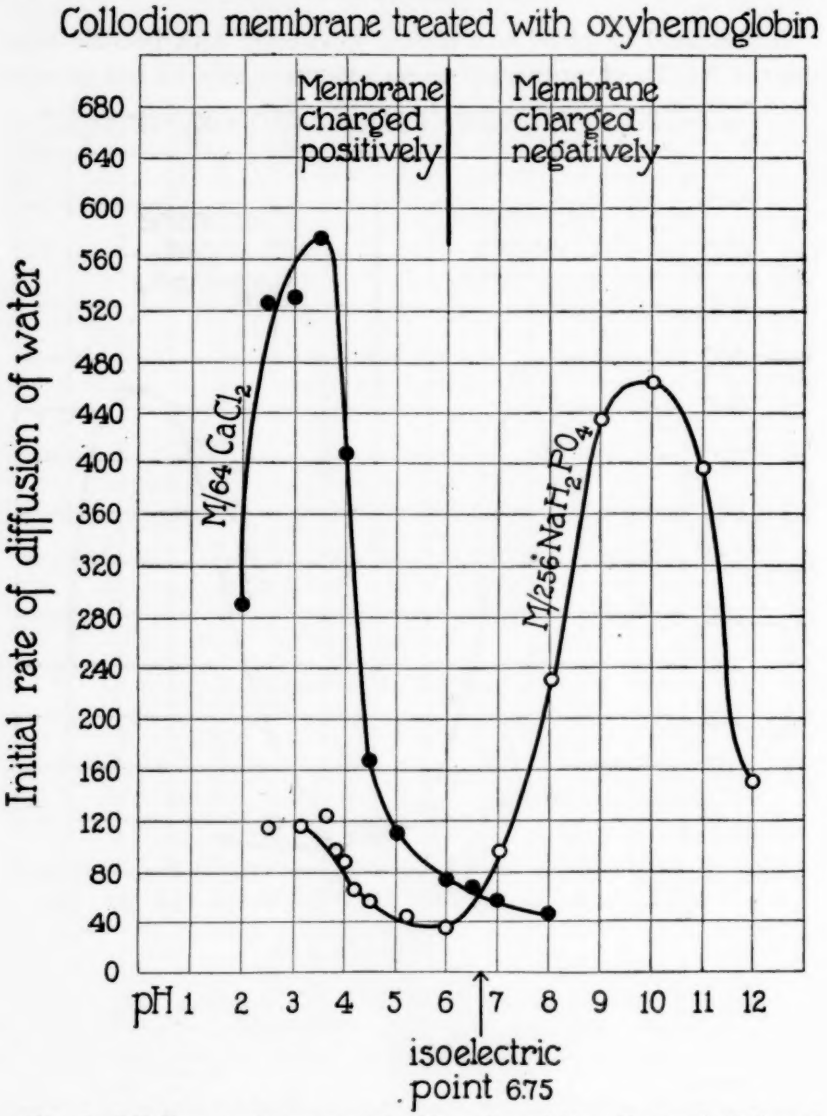

FIG. 6. *Collodion membranes treated with oxyhemoglobin.* Reversal of sign of charge at pH of about 6.0, on the acid side of the isoelectric point of oxyhemoglobin.

collodion bags were filled over night with this crude oxyhemoglobin solution and the bags were used the next day for similar experiments to those described for membranes treated with gelatin. Fig. 5 shows the results of such experiments. The $MgCl_2$ curve begins to rise at a pH of 6.0 while the curve for Na_2SO_4 rises at a pH of about 7.0 or 8.0. Hence when the membrane has been treated with oxyhemoglobin a much lower hydrogen ion concentration is required to induce a positive charge on the membrane than when the membrane has been treated with gelatin. This was to be expected if the reversal of the sign of charge of a membrane treated with a protein depends on the isoelectric point of the protein. According to Michaelis the isoelectric point of oxyhemoglobin is pH = 6.7 to 6.8, and we notice accordingly that the membrane treated with oxyhemoglobin assumes a positive charge when the pH is a little less than 6.7; *e.g.*, 6.0. In Fig. 6 the curve for NaH_2PO_4 shows a distinct rise at a pH of 6.0 and that for $CaCl_2$ at about 6.0 or a little less. In judging the curves in Figs. 5 and 6 the reader must bear in mind that the CO_2 of the air lowers the pH during the experiments and although the experiments lasted only 20 minutes the error so caused was noticeable in the neighborhood of the point of neutrality. Thus the beginning of the rise in the curve for Na_2SO_4 was in reality not at pH = 8, but at a lower pH, either 7.0 or probably less.

V. Collodion Membranes not Treated with a Protein.

When we use membranes *not* treated with gelatin and repeat the experiments described in Fig. 1, we get altogether different results (Fig. 7). There is no minimum or drop at pH of about 4.0 or 6.0 since the sign of charge of collodion membranes not treated with proteins is not reversed by acid.[3] Collodion membranes not treated with a protein are always negatively (and the watery phase positively) charged within the range of our experiments. Hence a M/256 solution of $CaCl_2$ cannot attract pure water through such a membrane even in acid solution and the curve for the effect of M/256 $CaCl_2$ is flat for the whole range of hydrogen ion concentrations (up to 10^{-2} N). The curve for M/256 Na_2SO_4 does not reach a minimum at pH 4.0 but drops slowly reaching a minimum at pH 2.0 or below, this drop being due to the depressing effect of the high concentration of the cat-

ions Na and H of the solution on the negative charge of the membrane. The drop of the curve at the alkaline end (pH = 10 to 12) is due to the high concentration of the cations Na and K, the latter introduced with the KOH added.

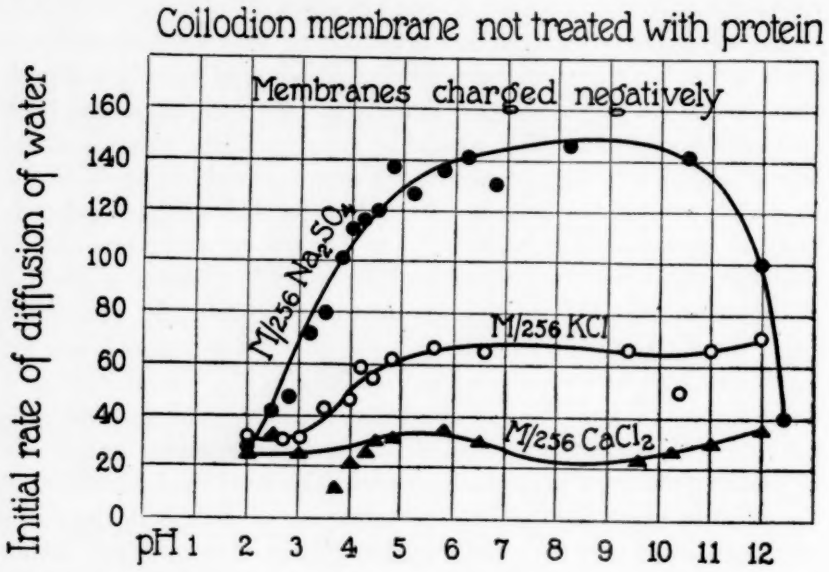

FIG. 7. *Collodion membranes not treated with protein.* No reversal of sign of charge of membrane.

VI. Theoretical Remarks.

Collodion membranes treated with oxyhemoglobin in the way mentioned remain red after they are washed, showing that the treatment of the membrane with a protein solution results in the formation of a durable protein film at the surface of the collodion membrane and probably also in the interstices of the membrane, since traces of oxyhemoglobin or at least of red pigment diffuse out of the membrane. Applying Werner's idea concerning the combination between acid and NH_3 to proteins we should expect that acids are added to an amino-acid group of a protein in a way similar to that in which they are added to NH_3; namely (if the acid added is HNO_3), by forming a salt of the type $R - N \begin{matrix} HNO_3 \\ H_2 \end{matrix}$ which dissociates into a positive $R - NH_3$ ion

and a negative NO_3 ion. On this assumption, the double layer at the boundary of the protein film and a solution would appear to be ionic in character, the positive stratum of the double layer being formed by the protein cations of the surface films of the membrane, while the mobile, watery stratum of the double layer contains the anions of the protein-acid salt. On this assumption it is clear why the hydrogen ion concentration required to make the membrane positive varies in the same sense as the isoelectric point of the protein used and why it is always higher than that of the isoelectric point, since a certain part of the surface of the protein film must be transformed into protein-acid salt before the membrane assumes a positive charge. At a hydrogen ion concentration below that of the isoelectric point the protein film is negatively charged since the protein exists here in the form of metal proteinate dissociating into a protein anion and a metal ion, the latter forming the positive watery stratum of the double layer, while the membrane owes its negative charge to the protein anion.

It does not follow, however, that this transformation of protein into protein-acid salt is the only, or even the essential, condition for the reversal of the sign of charge of the double layer by acid. Since acid can bring about a reversal in the electrical double layer of water bounded by air (or possibly even in a vacuum), it is obvious that the modification of the surface layer of the water which is in contact with the membrane may also play a rôle in the reversal of the sign of charge of the protein film. This possibility has to be considered also in view of the fact that trivalent or tetravalent cations can bring about a reversal in the sign of charge of a membrane treated with protein, even when the hydrogen ion concentration excludes a salt formation between protein and trivalent cation. These facts will be discussed in a subsequent paper.

SUMMARY.

1. It had been shown in previous papers that when a collodion membrane has been treated with a protein the membrane assumes a positive charge when the hydrogen ion concentration of the solution

with which it is in contact exceeds a certain limit. It is pointed out in this paper that by treating the collodion membrane with a protein (*e.g.* oxyhemoglobin) a thin film of protein adheres to the membrane and that the positive charge of the membrane must therefore be localized in this protein film.

2. It is further shown in this paper that the hydrogen ion concentration, at which the reversal in the sign of the charge of a collodion membrane treated with a protein occurs, varies in the same sense as the isoelectric point of the protein, with which the membrane has been treated, and is always slightly higher than that of the isoelectric point of the protein used.

3. The critical hydrogen ion concentration required for the reversal seems to be, therefore, that concentration where enough of the protein lining of the membrane is converted into a protein-acid salt (*e.g.* gelatin nitrate) capable of ionizing into a positive protein ion (*e.g.* gelatin) and the anion of the acid used (*e.g.* NO_3).